Evidence-Based Complementary and Alternative Medicine

Pharmacological Activities of Natural Products through the TGF-β Signalling Pathway

Lead Guest Editor: Chunpeng Wan
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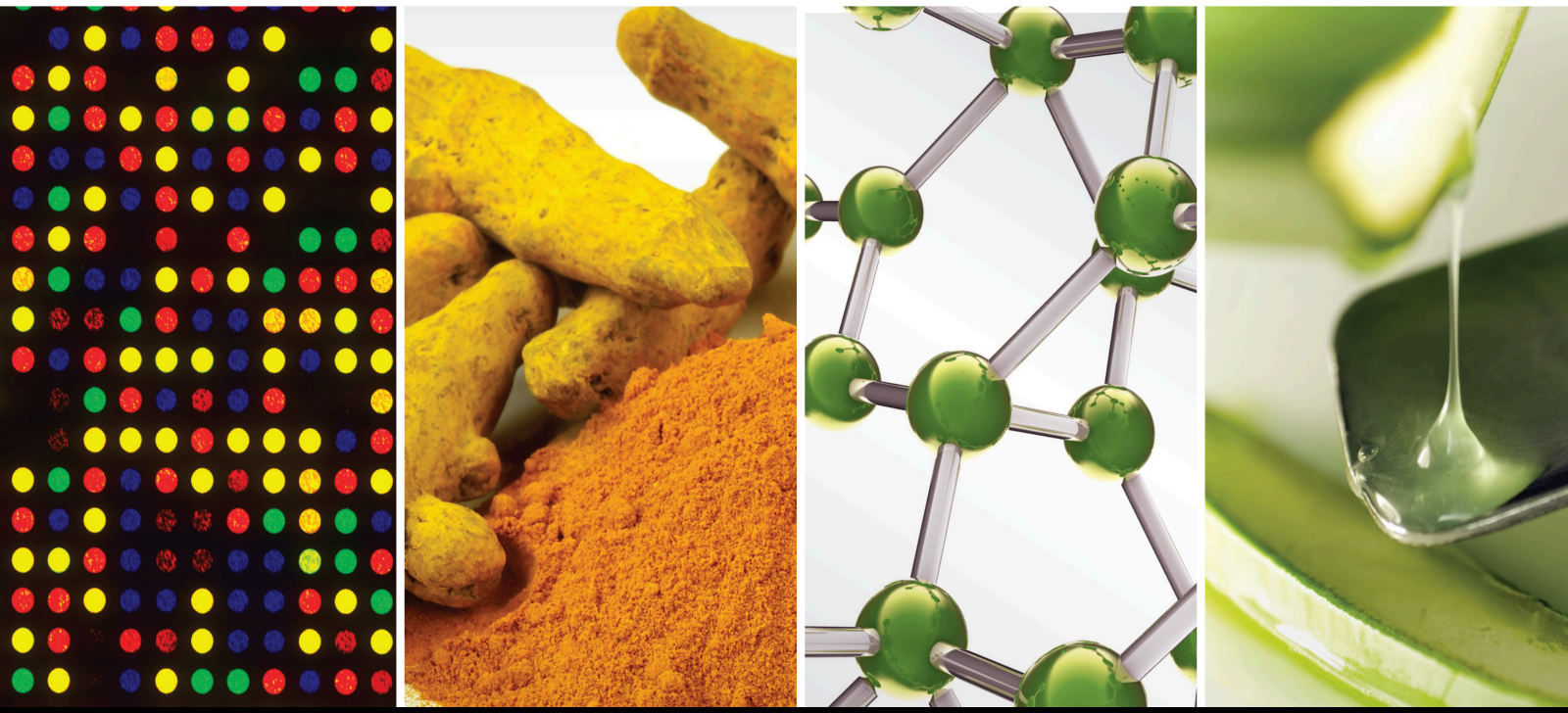

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Editorial Board

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Editorial

Pharmacological Activities of Natural Products through the TGF-β Signalling Pathway

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Transforming growth factor-β (TGF-β) belongs to the TGF superfamily, which is composed of TGF-βs and other related proteins, such as bone morphogenetic proteins (BMPs) and activins. By binding to a complex of receptor serine/threonine kinases at the cell surface, the ligands bind to the TGF-β receptors and mainly activate the phosphorylation of Smad proteins and promote their translocation into the nucleus with a common Smad4, followed by the transcriptional activation of target genes. In addition to the Smad-dependent pathway, the activated TGF-β receptors can also activate other signaling pathways, such as mitogen-activated protein kinase (MAPK) signaling pathway [1]. Activation of TGF-β signaling has diverse intracellular biological functions, including cell differentiation, cell proliferation, and migration, dependent on the downstream effectors. For example, in the context of carcinoma, TGF-β signaling suppresses tumor growth through induced cell cycle arrest and increased apoptosis but facilitates tumor cell dissemination *via* the production of prometastatic factors and epithelial-to-mesenchymal transition (EMT) in cancer cells. Therefore, TGF-β signaling and the corresponding cellular response are regarded as both a tumor-suppressive and a tumor-supportive event during cancer development and progression [2].

Based on the findings of TGF-β signaling and its regulatory effect in physiological and pathological processes, targeting of TGF-β signaling has been studied and applied in various disease diagnoses. Inhibition of the TGF-β1 and its downstream signaling has been reported to suppress the activity of myofibroblasts and excessive synthesis of extracellular matrix in the kidney, which significantly limits renal fibrosis during the progression of chronic kidney disease (CKD) [3]. Recently, targeting at the extracellular domain of TGF-β-neutralizing TGFBR2 can block the TGF-β signaling in T helper 2 (TH2)-cell and remodel the microenvironment of tumor cells in breast cancer [4]. Pharmacological blockade of TGF-β signaling is recognized as a potential therapeutic approach for the treatment of chronic fibrosis and different tumor entities.

This special issue provides a comprehensive study of natural products and herbal medicine targeting at TGF-β signaling pathway in a diversity of chronic diseases and cancers. For the special issue, the editorial office accepted six manuscripts for publication after peer-review process and selected four original research articles and two review articles with outstanding quality. The four original articles focused on the topic of this special issue, which is the pharmacological effect of natural products or herbal medicine targeting at the TGF-β signaling pathway. One review article emphasizes on the role of TGF-β signaling pathway and development of targeted therapy, while the other review provides an overview of the therapeutic effect of natural polyphenolic flavanone on the treatment of chronic diseases and malignancies.

The original paper "Antitumor Effects of Baicalein and Its Mechanism via TGFβ Pathway in Cervical Cancer HeLa

Cells" by G. Yu reported the therapeutic effect of baicalein, which was isolated from the Chinese herb of *Scutellaria baicalensis*, on the treatment of cervical cancer. The article illustrates the activity of baicalein on TGF-β signaling pathway blockage, overexpression of cell-adhesion molecule E-cadherin, and inhibition of EMT process in cervical cancer HeLa cells, which demonstrates the antimigratory effect of baicalein on cervical cancer. The original paper "Ginsenoside Rb3 Alleviates the Toxic Effect of Cisplatin on the Kidney during Its Treatment to Oral Cancer via TGF-β-Mediated Mitochondrial Apoptosis" by W. Wu assessed the activity of ginsenoside Rb3 in the oral cancer patients with cisplatin treatment. This article identified the protective effect of Rb3 on the activation of TGF-β signaling pathway and cellular apoptosis of kidney cells, determined by the cleavage of PARP and caspases driven by cisplatin administration, which consequently revealed the advantage of Rb3 pretreatment before chemotherapy in oral cancer patients. The research article "Punicalin Alleviates OGD/R-Triggered Cell Injury via TGF-β-Mediated Oxidative Stress and Cell Cycle in Neuroblastoma Cells SH-SY5Y" by T. Yang identified an active component from *Punica granatum* L. (punicalin) and analyzed its protective effect on oxidative stress of cells followed by TGF-β signaling activation during ischemia/reperfusion injury. In addition, the article "Study on Network Pharmacological Analysis and Preliminary Validation to Understand the Mechanisms of Plantaginis Semen in Treatment of Gouty Nephropathy" by H. Zhao screened for potential targets from public online databases and identified Plantaginis Semen as a potential inhibitor of TGF-β signaling in gouty nephropathy (GN). Activity of Plantaginis Semen was confirmed by the analysis of inflammatory factors, including TGF-β1, TNF-α, and IL-1β, as well as urinary protein levels in the treatment of GN.

In the review article "The Role of TGF-β Signalling Pathways in Cancer and Its Potential as a Therapeutic Target" by Y. Yang, the authors comprehensively discussed the multiple functions of TGF-β signaling and downstream effectors in different stages of tumorigenesis. The article gives an overview of the tumor-suppressive role of TGF-β signaling through the cell cycle arrest and tumor cell apoptosis and, meanwhile, the prometastasis activity of TGF-β signaling in the reestablishment of tumor-favorable microenvironment. Moreover, the review article also summarized the therapeutic components targeting at TGF-β family ligands or TGF-β receptors, including antisense oligonucleotides and monoclonal antibodies. The other review article "Pharmacological Activity of Eriodictyol: The Major Natural Polyphenolic Flavanone" by Z. Deng gives insight into the flavonoid eriodictyol with negative regulatory effect of PI3K/Akt signaling pathway, matrix metalloproteinases (MMPs) expression, and increased cell apoptosis, which highlights the therapeutic effect of eriodictyol in the protection of cell injury as well as the treatment of malignant tumors.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

Acknowledgments

We appreciate the authors and contributors of this special issue for their efforts to their valuable papers. At the same time, we would like to thank the reviewers for their timely response and constructive suggestions to the articles. Lastly, we would like to give our sincere thanks to the Editorial Board for inviting us to edit this special issue. We hope the special issue will give informative knowledge for understanding the TGF-β signaling pathway and pharmacological activities of natural products targeting at the TGF-β signaling.

Chunpeng (Craig) Wan
Muhammad Farrukh Nisar
Hua Wu

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Review Article

The Role of TGF-β Signaling Pathways in Cancer and Its Potential as a Therapeutic Target

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The transforming growth factor-β (TGF-β) signaling pathway mediates various biological functions, and its dysregulation is closely related to the occurrence of malignant tumors. However, the role of TGF-β signaling in tumorigenesis and development is complex and contradictory. On the one hand, TGF-β signaling can exert antitumor effects by inhibiting proliferation or inducing apoptosis of cancer cells. On the other hand, TGF-β signaling may mediate oncogene effects by promoting metastasis, angiogenesis, and immune escape. This review summarizes the recent findings on molecular mechanisms of TGF-β signaling. Specifically, this review evaluates TGF-β's therapeutic potential as a target by the following perspectives: ligands, receptors, and downstream signaling. We hope this review can trigger new ideas to improve the current clinical strategies to treat tumors related to the TGF-β signaling pathway.

1. Introduction

The transforming growth factor-β (TGF-β) was first discovered in 1978 by JE de Larco and GJ Todaro in mouse fibroblasts transformed with murine sarcoma virus. TGF-β is a member of the cell growth factor superfamily; it is involved in the regulation of various biological processes, including cell growth, differentiation, autophagy, apoptosis, epithelial-mesenchymal transition (EMT), angiogenesis, inflammation, and immunity [1–5]. TGF-β mainly exerts multiple biological functions in the body through two pathways: the classic SMAD-dependent pathway and the non-SMAD-dependent pathway. In the SMAD-dependent classical pathway, there are two transmembrane Ser/Thr kinase receptors in the cell membrane, namely, TGF-β receptor I (TβR I) and TGF-β receptor II (TβR II). The combination of TGF-β and TβR II can activate the kinase activity of TβR I and induce the phosphorylation of TβR I. Subsequently, the activated TβR I can recruit and phosphorylate downstream SMAD proteins, SMAD2 and SMAD3. Once phosphorylated, SMAD2 and SMAD3 bind to the chaperone protein SMAD4 and are cotransported to the nucleus, where they can regulate the expression of TGF-β target genes [6, 7] (Figure 1). Here, we mainly discuss the related role of TGF-β in tumors and its potential as a therapeutic target. We first introduce the related role of this signaling pathway in tumorigenesis and development. Then, using the classic TGF-β signaling pathway as a framework, we discuss the molecules and mechanisms that cause the abnormal activation or inactivation of TGF-β from three perspectives. Finally, we summarize the current TGF-β-targeted tumor therapy drugs from these three perspectives. We hope that readers can expand the idea of designing new TGF-β tumor treatment drugs.

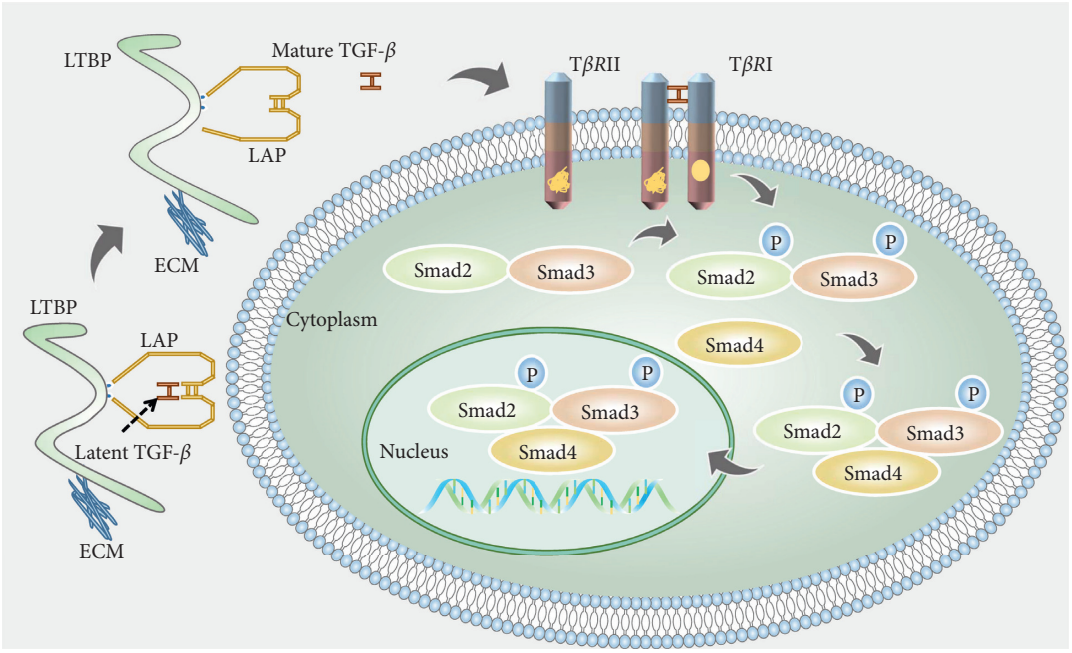

Figure 1: Mature TGF-β ligand, LAP, and LTBP together form a large latency complex that keeps the TGF-β ligand in a latent state at this time. When the TGF-β ligand is released from the complex, it changes from a latent to an active state. The released TGF-β ligand can directly bind to TβR II, thereby further binding to TβR I, but TGF-β ligand cannot directly bind to TβR I. The combination of TGF-β ligand, TβR II, and TβR I can further transmit signals to downstream mediators. After phosphorylation and activation of SMAD2 and SMAD3, they further bind to SMAD4 and transmit the signal to the nucleus.

2. Introduction to the Related Mechanisms and Functions of TGF-β Signaling Pathway in Tumorigenesis and Development

For the tumor, TGF-β is a double-edged sword, as it can achieve inhibition and promotion of tumors through various mechanisms (Figure 2). TGF-β has a strong cellular inhibitory ability and is a prominent antiproliferation agent. It can inhibit cell cycle progression by blocking the G1 phase and exert its antiproliferation ability by inhibiting proliferation drivers such as C-MYC and ID [8, 9]. TGF-β can also induce apoptosis to inhibit tumor growth [10]. In addition to acting directly on epithelial tumor cells, TGF-β can further control tumor development by regulating the production of growth factors in the surrounding stroma and the tumor microenvironment [8]. Furthermore, TGF-β inhibits inflammatory and immune processes [11]. However, when the immunosuppressive action of TGF-β becomes significant, it will eventually start promoting tumor progression. TGF-β inhibits the transcription of proapoptotic and lysogenic cytokines in cytotoxic T lymphocytes (CTLs), such as perforin, Granzyme A (GZMA), Granzyme B (GZMB), porphyrin interferon g

, and factor-associated suicide (FAS) ligands [12, 13]. TGF-β inhibits certain functions of CTLs, $CD8^+$ T cells, and natural killer cells, resulting in a tumorigenic effect [14, 15]. TGF-β also enhances tumor invasiveness and angiogenesis by promoting the production and secretion of matrix metalloproteases proteinase-2 (MMP-2) and matrix metalloproteinase-2 (MMP-9) and downregulating the expression of tissue inhibitors of metalloproteases (TIMP) [8, 16–18]. TGF-β also induces EMT, which supports tumor invasion and spread by releasing tumor cells into the environment and promoting their movement [16].

2.1. Tumor Inhibition by TGF-β

2.1.1. TGF-β Inhibits Tumors by Regulating Cell Proliferation. TGF-β inhibits cell proliferation primarily through two transcriptional events: the induction of cyclin-dependent kinase (CDK) inhibitors and C-MYC expression inhibition [19]. In neuronal, epithelial, and hematopoietic cells, TGF-β inhibits cell growth by targeting CDKs and their inhibitors (CDK-IS), responsible for controlling cell cycle progression beyond G1 during proliferation. P15INK4B, P21CIP1, and P27KIP1 are three CDK-IS whose expression is promoted by TGF-β, which also inhibits the cyclin-CDK complex, leading to cell cycle arrest of G1 phase [19–22]. P15 mainly blocks the interaction between CDK4/6 and cyclin D, thus inhibiting the cell cycle process in the late G1 phase [23]. As a CDK inhibitor, P27 can be removed from the cyclin D-CDK4 complex, then interact with the cyclin E-CDK2 complex, and inhibit the cyclin E-CDK2 complex. P21 can also inhibit the activity of the cyclin E-CDK2 complex [9, 23]. When these CDK complexes are inactive, retinoblastoma protein (pRb) phosphorylation is inhibited, and pRb phosphorylation is the main switch in cell cycle progression, thus preventing G1 cells from moving into the S phase. Simultaneously, TGF-β can downregulate C-MYC oncogene expression, thereby inhibiting cell proliferation.

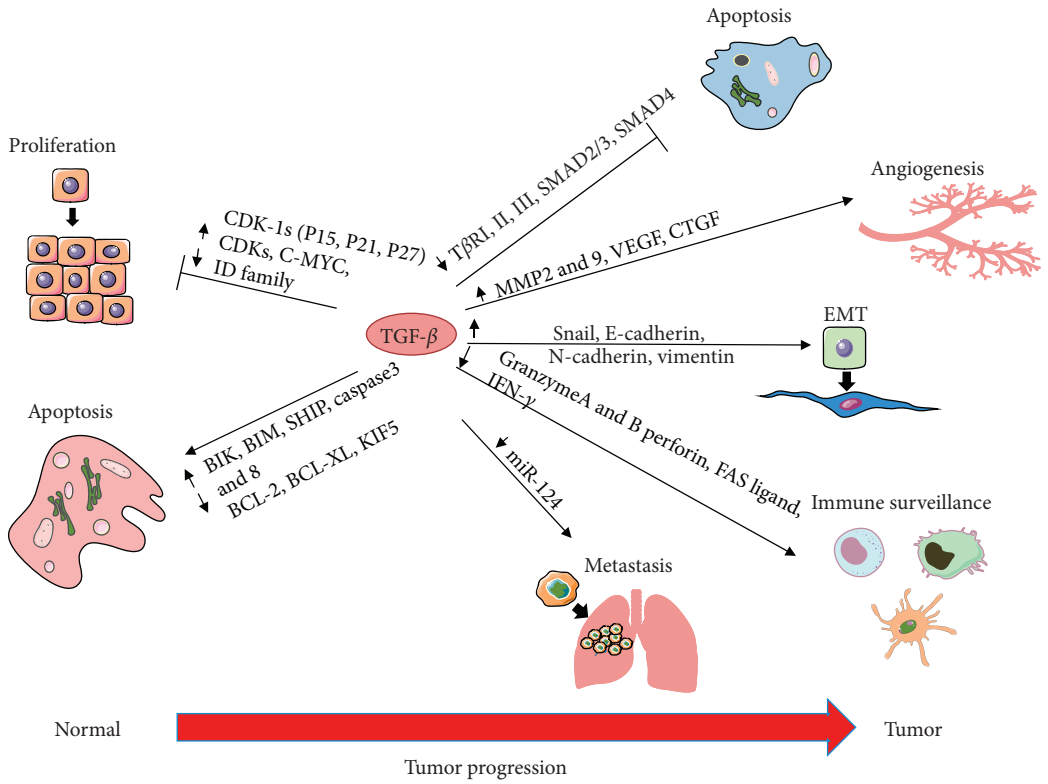

Figure 2: Dual effects of TGF-β on tumors. As a double-edged sword, TGF-β can promote and inhibit tumors through various mechanisms. TGF-β exerts its tumor inhibition mainly by inhibiting cell proliferation and inducing apoptosis. TGF-β can upregulate CDK-IS expression (P15, P21, and P27) to inhibit CDK and downregulate the expression of the C-MYC and ID family to inhibit cell proliferation. Simultaneously, TGF-β can also inhibit the expression of antiapoptotic genes such as BCL-X, BCL-2, and KIF5 and promote the expression of proapoptotic genes such as BIK, Caspase 3, and Caspase 8 to induce apoptosis and thus inhibit tumor growth. However, TGF-β can also promote cancer through several mechanisms. TGF-β can enhance EMT and metastasis to play its protumor role by upregulating Snail, E-cadherin, and N-cadherin or downregulating miR-124. It can also evade the immune system by inhibiting Granzyme AB, perforin, FAS ligands, and IFN-γ to achieve its tumor-promoting effects. TGF-β also triggers tumor growth by promoting angiogenesis by activating MMP2, MMP9, VEGF, and CTCT.

C-MYC allows cells to multiply indefinitely and promotes cell division. In epithelial cells, the TGF-β-induced SMAD complexes synergistically regulate C-MYC expression's downregulation with transcription factors P107, E2F4/E2F5, and CCAAT/enhancer-binding protein (C/EBP) [24]. In addition, TGF-β inhibits Id1, Id2, and Id3 expression, which are nuclear factors associated with the G1 to S cell cycle transition. Inhibition of Id family proteins by TGF-β leads to decreased proliferation [25, 26]. The SMAD-dependent pathway of TGF-β is also associated with antiproliferative responses [27]. For example, TGF-β inhibits P70 S6 kinase by protein phosphatase 2A (PP2A) and induces G1 phase cell cycle arrest [28].

2.1.2. TGF-β Inhibits Tumors by Promoting Apoptosis. TGF-β can trigger apoptosis of various cell types to inhibit tumor growth; there are two main pathways: the SMAD-dependent pathway and the independent pathway. However, the molecular mechanisms are still less clear. The SMAD-dependent pathway involves proapoptotic proteins such as death-related protein kinases (DAPK), Src homology inositol phosphatase (SHIP), and TGF-β induced early gene 1(TIEG1). Among them, DAPK can promote the release of cytochrome C and mediate TGF-β-dependent cell apoptosis by associating SMADs with mitochondrial proapoptotic events [29]. SHIP inhibits the PI3K-Akt pathway leading to cell death before cell survival [30]. TIEG1 can induce oxidative stress and produce reactive oxygen species (ROS) [31, 32]. These all promote apoptosis and thus inhibit tumor growth, and the expression of these proapoptotic proteins is regulated by TGF-β-mediated SMAD signaling. The DPC4-induced SAPK/JNK signaling pathway is also involved in TGF-β signaling, which leads to apoptosis [33]. In the TGF-β independent pathway, TGF-β-mediated apoptosis is involved in the activation of caspase. TGF-β inhibits the expression of antiapoptotic genes such as BCL-2 family members, BCL-XL, and X-linked inhibitor of apoptosis (XLAP) and promotes the expression of some proapoptotic genes such as caspase 3, caspase 8, and Bcl-2-interacting killer (BIK) [34–36]. Death domain-associated protein (DAXX) is a protein associated with the FAS receptor and is associated with the apoptotic signal of TGF-β. DAXX is involved in TGF-β-mediated JNK activation, thereby mediating programmed cell death [37]. TGF-β has been reported to increase the expression of the death-related

protein kinase DAPK in liver cancer cells and signal transduction factors 45β for growth stagnation and DNA damage (GADD45beta) in the liver cells [38]. In hepatocytes, TGF-β induces cell death by producing ROS [39]. The production of TGF-β-induced ROS promotes apoptosis by regulating various members of the BCL-2 family, such as BCL-2 modifying factor (BMF) and BCL-2 interacting mediator (BIM) [40]. In gastric cancer cell lines, TGF-β mediates physiological apoptosis of gastric epithelial cells by activating apoptotic molecules BIM and Caspase 9 [41]. In pancreatic ductal adenocarcinoma (PDAC), TGF-β inhibits the expression of major gastrointestinal spectrum regulator, Krüppel-like Factor 5 (KLF5). However, KLF5 and SRY-related hug box 4 (SOX4) have synergistic effects, and the inhibition of KLF5 promotes apoptosis in the SOX4 program [42]. The mechanism by which TGF-β promotes the apoptotic responses that inhibit the tumors remains to be investigated. Further insights could provide new strategies for tumor inhibition and treatment.

2.2. Tumor Promotion by TGF-β

2.2.1. Tumor Promoter Role of TGF-β in EMT. The EMT of tumor cells is a crucial step in tumor metastasis. EMT is essential in wound healing, fibrosis, cancer progression, and embryonic development [43]. TGF-β induces EMT during average growth and development. TGF-β-induced EMT supports tumor invasion and spread by releasing tumor cells into the environment and promoting their movement. In many cancers, TGF-β induces EMT with the transcriptional regulation of E-cadherin, N-cadherin, Snail, and vimentin [44, 45]. TGF-β and adhesion-dependent signaling are required for stable expression of myofibroblast phenotypes to induce cytoskeletal recombination [46]. After EMT, epithelial cells lose their polarity, tight junctions, and adhesion between cells, thus gaining the ability to migrate. This phenotypic change leads to reduced intercellular adhesion and enhanced migration and invasion ability of tumor cells, thus promoting cancer metastasis [47]. In breast cancer, the developmental transcription factor SOX4 can mediate TGF-β-induced action and promote EMT, tumor progression, and metastasis in breast cancer [48]. Moreover, the expression of TGF-β can also induce double mouse minute 2 (MDM2) expression, which makes p53 unstable, leading to EMT and tumor progression [49]. In p53-mutated cancers, TGF-β induces the assembly of the mutant p53, p63 protein complex, and SMADs. In this ternary complex, the tumor-suppressive function of P63 is antagonized, and the inactivation of P63 enabled both the mutant p53 and TGF-β to initiate EMT [50]. In addition, various studies have shown that TGF-β is involved in the EMT of tumor cells and the invasion and metastasis of a tumor cell. TGF-β promotes prostate cancer migration by inducing stress fiber aggregation and cytoskeletal rearrangement through the cell division cycle 42 (Cdc42), Rho A, and SMAD proteins [51]. TGF-β also induces the expression of dedicated for cytokinesis 4 (DOCK4) protein through the SAMD pathway, enhancing the exudation of lung cancer tumor cells and increasing the motility metastasis of tumor cells [52]. In non-small-cell lung cancer, activation of the TGF-β pathway leads to a severe loss of *miR-124*, enhancing EMT and metastasis [53]. TGF-β-driven EMT gives cancer cell motility, metastasis, and progenitor cell-like characteristics, all of which enable TGF-β to play its tumor-promoting role.

2.2.2. Tumor Promoter Role of TGF-β in Angiogenesis. During tumor growth, the vascular network's development is essential because the proliferation and metastasis of tumor cells require nutrition and oxygen, which requires more angiogenesis. The expression level of angiogenic factors also reflects the invasion ability of the tumor [54]. Endothelial cells (EC) play a crucial role in angiogenesis. EC showed higher cell proliferation, migration, and invasion during neovasculature, and TGF-β signaling complexly correlates with EC ability and activity [55]. TGF-β also induces proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF), through fibroblasts and epithelial cells. These factors directly stimulate EC to form capillaries and play an essential role in inducing and maintaining tumor angiogenesis, thus accelerating cancer progression [56, 57]. Simultaneously, TGF-β can induce endothelial migration, which is necessary for angiogenesis [58, 59]. In liver cancer, prostate cancer, and renal cell carcinoma, high plasma levels of TGF-β are also associated with increased tumor angiogenesis and poor prognosis in these cancers [60, 61]. In non-small-cell lung cancer, the higher level of TGF-β in the tumor microenvironment is associated with increased angiogenesis, tumor progression, and poor prognosis [62]. In human breast cancer, the high mRNA levels of TGF-β are associated with increased microvascular density and these parameters are related to patients' poor prognosis [63]. TGF-β/SMAD4 signaling can upregulate the expression of miR-29a, which can target phosphatase and tensin homolog (PTEN) and activate the AKT pathway, thereby promoting the generation of new blood vessels [64]. In addition to TGF-β ligand action, TGF-β receptors are also critical for angiogenesis. TGF-β can enhance the expression of MMP9 and promote the formation of new blood vessels through one of its type I receptors, ALK5. TGF-β-ALK5 signal transduction can enhance the angiogenesis and invasiveness of breast cancer cells and prostate cancer cells [65, 66].

2.2.3. Tumor Promoter Role of TGF-β in Immunologic Surveillance. TGF-β plays a systemic immune role and can significantly inhibit tumor immune surveillance of the host. TGF-β inhibits cytotoxic T cells, dendritic cells, and natural killer (NK) cells and produces a proinflammatory environment [67]. Cytotoxic $CD8^+$T cells produce many cytokines, including perforin, GZMA, GZMB, IFN-γ, and FASL, which induce apoptosis of cancer cells. However, TGF-β can inhibit the expression of these cytotoxic genes through SMADs and ATF1. The neutralization of TGF-β in vivo restores the expression of critical cytotoxic genes involved in tumor clearance, thereby promoting the removal of antigen-specific tumors in vivo. These all suggest that TGF-β directly

targets cytotoxic T cells to play its prooncogenic role during tumor evade immune surveillance [13]. Dendritic cells (DCs) are antigen-presenting cells responsible for inducing adaptive T cell response, and their activity has essential significance in antitumor immunity [68, 69]. TGF-β upregulates the differentiation inhibitor of TGF-β, Id1, and the overexpression of Id1 downregulates key factors of DC differentiation, leading to systemic immunosuppression [70]. NK cells also play an essential role in immune surveillance by directly recognizing tumor cells and initiating cytotoxic reactions [71]. TGF-β inhibits NK cell activation by diminishing the production of IL-15 and downregulating its active receptor natural killer group 2, member D (NKG2D) [72, 73]. In human glioma, TGF-β reduces the expression of NKG2D in $CD8^+$ T and NK cells and inhibits the expression of the MICA, which is the ligand of NKG2D [74]. In addition to lymphocytes, TGF-β also has significant effects on some myeloid cells, which mainly consist of two myeloid cell types, namely, tumor-associated macrophages (TAM) and tumor-associated neutrophils (TAN). There are two phenotypes of TAM. The classically activated M1 phenotype can inhibit tumor growth, while the nonclassically activated M2 phenotype can promote tumor growth. TGF-β primarily drives the differentiation of the M2 phenotype of macrophages. They produce many different cytokines, such as MMP9, C-X-C motif ligand 8 (CXCL8), and IL-10, which can induce tumor growth and development [75]. Like TAM, TAN also has two phenotypes: antitumor phenotype (N1) and tumorigenic phenotype (N2). It has been shown that in the presence of TGF-β, neutrophils develop into an N2 phenotype that is not cytotoxic to the tumor. In the N2 phenotype, TAN's ability to secrete antitumor cytokines and activate cytotoxic T cells decreases, contributing to tumor growth and immunosuppression [76].

3. Introduction to the Molecules and Mechanisms That Regulate the TGF-β Signaling Pathway

As mentioned above, TGF-β can inhibit tumor occurrence by inhibiting cell proliferation and promoting cell apoptosis and tumor invasion and metastasis by inducing EMT, inducing angiogenesis, and inhibiting immunity. The TGF-β signaling pathway is precisely regulated under normal physiological conditions. Therefore, once the TGF-β signaling pathway is abnormally activated or blocked, this balance will be struck, aiding in the development of tumors. Next, we discuss some molecules and mechanisms that can activate or inhibit the TGF-β signaling pathway. We divide the molecules and mechanisms that regulate the TGF-β signaling pathway into the following three perspectives.

3.1. Regulation of the TGF-β Signaling Pathway at the Levels of the Ligands. Most TGF-β ligands exist in a latent state in the body, and the latent TGF-β binds noncovalently to the C-terminal prodomain latency-related peptide (LAP) to form a small latency complex (SLC). This small complex can further bind to the incubation period TGF-β binding protein 1 (LTBP1), and the three form a large latent complex (LLC) [77]. While being part of this structure, TGF-β cannot bind to its receptor and cannot exert its biological activity. It can only be connected to the extracellular matrix's binding site through LTBP [78]; in other words, TGF-β is latent. The process of transforming TGF-β from a latent state to an active state is called ligand activation. The TGF-β ligand must undergo activation to exert its biological activity. This feature also makes the regulation of the TGF-β activation process a critical point in regulating the TGF-β signaling pathway from the perspective of the ligand. The TGF-β ligand in the large latency complex cannot bind to the corresponding receptor. Therefore, the TGF-β ligand must be released from the large latency complex to make TGF-β active. The TGF-β ligand can be removed and activated from the complex in the following four ways.

(1) Exposure to specific physical or chemical conditions, such as heat shock, extreme pH changes, ionizing radiation, and physical shearing force, can promote the separation of large complexes and activate TGF-β ligands [79–84].

(2) Activation by enzymatic forms, including many different types of proteases, such as aspartic, cysteine, metalloproteinases, serine proteases, and neuraminidase expressed on the viral particles' surface, can release TGF-β ligands by inducing conformational changes in the latent complex [81, 85–89].

(3) Some factors mainly act on LAP to activate TGF-β, such as ROS, thrombospondin 1 (TSP1) and members of the αv integrin family (including $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha v\beta 6$); these substances can release the noncovalent binding between LAP and TGF-β by acting on LAP, thereby releasing mature TGF-β [90–92].

(4) Some substances can act on LTBP to activate TGF-β. From the previous description of the latent state of TGF-β, we can see that to release TGF-β from the complex, one way is to act on LAP, and the other is to act on LTBP. Most substances promote the activation of TGF-β by working on LAP. The three pathways we mentioned above can be summarized as using LAP as the point of action to activate TGF-β and LTBP as the trigger of TGF-β activation is relatively rare. Still, bone morphogenetic protein 1-(BMP1-) like protease can activate TGF-β by directly cleaving LTBP1 [93]. The substances that regulate the TGF-β signaling pathway at the level of the ligands include the ligands that activate TGF-β and the ligands that can inhibit the activation of the latent state TGF-β, such as the LSKL peptide, which is a competitive antagonist. The LSKL peptide inhibits the activation of TGF-β by preventing the interaction of TSP1 with the LAP of potential TGF-β [94], such as Emilin1, a cysteine-rich secreted glycoprotein expressed in the vascular tree. Emilin1 can inhibit TGF-β signaling by specifically binding to TGF-β to prevent TGF-β maturation [95]. Another example is

Cripto, a developmental cancer protein that can prevent the TGF-β ligand from binding to the receptor by binding to the TGF-β ligand, thereby inhibiting TGF-β signaling [96].

3.2. Regulation of the TGF-β Signaling Pathway at the Level of the Receptors. All TGF-β ligands can bind to and activate the heterologous cell surface complex of their receptors. These receptors can be divided into TβR I and TβR II based on sequence similarity. Both TβR I and TβR II contain a serine/threonine kinase active transmembrane receptor [97]; the receptor structure can be divided into the extracellular domain and a transmembrane intracytoplasmic domain. In the intracytoplasmic region of TβR I, a highly conserved GS region (an area rich in glycine and serine residues) is the active region of TβR I kinase. This region can be phosphorylated by TβR II [98]. Although TβR II and TβR I are structurally similar, TβR II does not have a GS region but a short tail rich in serine and threonine at the hydroxyl end of the intracytoplasmic part. The TGF-β ligand itself cannot bind to TβR I. It can only attract TβR I to the cell surface after binding to TβR II and promote the phosphorylation of TβR I through TβR II. This staged process is essential for the smooth transmission of TGF-β signals. Therefore, the regulation from the receptors' perspective is another vital entry point for regulating TGF-β signaling pathways. Here, according to the regulatory mechanism, the TGF-β signaling pathway regulation from the receptor's perspective is divided into the following two ways.

3.2.1. Regulatory Pathways Associated with Posttranslational Modification of Receptors. As mentioned above, the combination of TβR II and TβR I can promote the phosphorylation of TβR I. Phosphorylation of TβR I is an essential basis for TGF-β signal transduction. Only phosphorylation of TβR I can further activate downstream signaling mediators of the TGF-β signaling pathway, SMAD2, and SMAD3. Therefore, substances that affect receptor phosphorylation can activate or inhibit the TGF-β signaling pathway. For example, protein phosphatase 1 (PP1) can dephosphorylate TβR I to inhibit TGF-β signaling, SMAD anchor for receptor activation protein (SARA) can recruit the catalytic subunit of PP1 to dephosphorylate the receptor to inhibit the TGF-β signaling pathway [99], and 12 kDa FK506-binding protein (FKBP12) can bind to the GS domain of TβR I, thereby inhibiting the phosphorylation of TβR I [100]. Interestingly, SMAD7 can act on two posttranslational modification regulatory pathways. It can dephosphorylate and inactivate the receptor, and it can also induce receptor degradation by recruiting an E3 ubiquitin ligase. Eventually, the receptor is inactivated by dephosphorylation and is degraded by ubiquitination [101]. In addition to phosphorylation, ubiquitination is also a common posttranslational modification of TGF-β receptors. Therefore, substances that affect receptor ubiquitination can also regulate the TGF-β signaling pathway. SMAD7, FKBP12, and neural precursor cell expressed developmentally downregulated 4-like (NEDD4-2) also degrade the receptor by promoting its ubiquitination [102]. In contrast, C-CBL, heat shock protein 90 (Hsp90), transforming growth factor-beta stimulated clone 22 (TSC-22), tumor necrosis factor receptor-associated factor 4 (TRAF4), ubiquitin-specific protease 4 (USP4), ubiquitin-specific protease 11 (USP11), ubiquitin-specific protease 15 (USP15), and UCH37 can stabilize the receptor by blocking the ubiquitination of the receptor [103–110], thereby activating the TGF-β signaling pathway.

3.2.2. Other Regulations besides the Posttranslational Modification. Regarding the regulation of the TGF-β signaling pathway at the level of the receptor, in addition to the posttranslational modification of the receptor, there are some factors or proteins that can directly interact with the receptor, causing the receptor to degrade and block signal transduction or after binding to the receptor, thus preventing the binding between the receptor and receptor, the binding between the receptor and ligand, and the binding between the receptor and the downstream players. Among others, these factors include toll-interacting protein (TOLLIP), salt-inducible kinases (SIK), caveolin-1 (CAV-1), Dapper 2 (dvl-associated proteins), and protein interacting with c-kinase 1 (PICK1). Their binding to the TGF-β receptor promotes receptor degradation, thereby inhibiting the TGF-β signaling pathway [111–115]. Other examples are BMP and activin membrane-bound inhibitor (BAMBI), FKBP12, serine-threonine kinase receptor-associated protein (STRAP), C-SKI (a transcriptional corepressor of SMAD-dependent TGF-βsignaling), DRAK2 (A serine/threonine kinase belonging to the death-associated protein kinase family), ventricular zone-expressed pH domain-containing 1 (VEPH1), and additional substances that can bind to TGF-β receptors, thereby interfering with the binding of receptors to factors required for normal signal pathway transduction [116–120]. Of course, in addition to these factors (or proteins) that bind to receptors that can antagonize TGF-β signaling, some factors (or proteins) can interact with receptors to promote signaling. For example, 14-3-3ε, 14-3-3ε is the first protein other than SMADs that has been confirmed to interact with TGF-β receptors and activate signal transduction. It can interact with TβR I to induce TGF-β-induced signal transduction [121, 122]. For instance, the Bα subunit of protein phosphatase 2A, the Bα regulatory subunit, can interact with the cytoplasmic domain of TβR I to promote signal transduction [123]. Another example is disintegrin and metalloproteinase 12 (ADAM12). ADAM12 can bind to TβR II and stabilize the receptor by controlling the localization of the TGF-β receptor to the early endosome, thereby enhancing TGF-β signaling [124].

3.3. Regulation of the TGF-β Signaling Pathway at the Level of the Downstream Signaling. SMADs are crucial downstream signaling mediators of the TGF-β signaling pathway. The primary function is to transmit TGF-β signals from the cell membrane to the nucleus, thereby regulating the corresponding target genes' transcription and expression. Based on functional differences in the classic TGF-β signaling pathways, the SMAD proteins can be divided into three

types. The first type includes the receptor-regulated SMADs, SMAD2, and SMAD3, which can be activated by TβR I-induced phosphorylation. The second is the universal SMAD, that is, SMAD4. SMAD4 can interact with SMAD2 and SMAD3 to help both transmit signals to the nucleus. The third includes the inhibitory SMADs, SMAD6, and SMAD7. The inhibitory SMAD proteins can negatively regulate the TGF-β signaling pathway through various mechanisms of action. Regardless of the SMAD protein, its activation or inhibition can affect TGF-β signal transduction, which is of great significance for TGF-β signal transduction.

3.3.1. Regulatory Pathways Related to Posttranslational Modification of SMADs Protein. The posttranslational modification of the SMADs protein is the same as the posttranslational modification of the receptor, i.e., phosphorylation and ubiquitination. SMAD2 and SMAD3 can be phosphorylated and be further activated by TβR I, which means that the phosphorylation of SMAD2 and SMAD3 is necessary for the smooth transmission of the TGF-β signaling pathway. Thus, some factors or proteins that regulate the phosphorylation of SMAD2 and SMAD3 may influence the TGF-β signaling pathway, such as liver fibrosis-associated lncRNA1 (lncLFAR1) and lysyl oxidase-like 1 (LOXL1) [125, 126], both of which bind to SMAD2 and SMAD3 and promote their phosphorylation in the cytoplasm to activate the TGF-β signaling pathway, thereby stimulating the development of liver fibrosis. Some substances can inhibit the phosphorylation of these two SMADs by interacting with SMAD2 and SMAD3, such as protein phosphatase, Mg^{2+}/Mn^{2+}-dependent 1A (PPM1A), protocadherin gamma-A9 (PCDHGA9), heat shock protein 70 (Hsp70), and calcium-sensitive receptor (CaSR), ultimately inhibiting the conduction of the TGF-β signaling pathway by inhibiting the phosphorylation of SMAD2 and SMAD3 [127–130]. The phosphorylation of SMAD2 and SMAD3 activates TGF-β signaling, whereas ubiquitination and degradation of SMAD2 and SMAD3 inhibit TGF-β signaling transduction. For example, AXIN, DREB, and EAR motif protein 1 (DEAR1) can inhibit the TGF-β signaling pathway's conduction by promoting the degradation of SMAD3 ubiquitination [131, 132]. In contrast, OTU domain, ubiquitin aldehyde binding 1 (OTUB1), B-cell lymphoma-3 (BCL-3), ubiquitin carboxyl-terminal hydrolase 1 (UCHL1), and UCHL5 contribute to the deubiquitination of SMAD2 or SMAD3, making them more stable and less easily degradable and promoting TGF-β signaling [133–136]. The regulation of the TGF-β signaling pathway from the perspective of downstream signaling mediators includes not only SMAD2 or SMAD3 but also the regulation of universal SMAD and inhibitory SMAD, such as the wild-type p53-induced phosphatase 1 (Wip1), which selectively binds SMAD4 and dephosphorylates it, thereby inhibiting TGF-β signaling [137]. Examples include ubiquitin-specific protease 10 (USP10), which can act on SMAD4 to make it deubiquitinated and stable, further promoting TGF-β signaling [138], and USP26, which promotes SMAD7 deubiquitination, thereby amplifying the inhibitory effect of SMAD7 and strengthening the inhibition of the TGF-β signaling pathway [139].

3.3.2. Regulatory Mechanisms beyond Posttranslational Modifications. In this section, in addition to the regulation of downstream media through posttranslational modification, we discuss two forms of regulation that enhance or hinder the synergism between SMADs by binding to SMADs to affect the entry of SMADs into the nucleus.

By affecting SMAD proteins' entry into the nucleus, players such as miR-26a, IL-6 (interleukin-6), and HSP72 can block the downstream signaling events of TGF-β by inhibiting the nuclear translocation of phosphorylated SMAD proteins [140–142]. Another example is PCDHGA9, a member of the cadherin family that inhibits not only the phosphorylation of SMAD2/3 but also the nuclear translocation of pSMAD2/3, inhibiting downstream signaling events of TGF-β through a dual-action. Compared to the number of substances that inhibit nuclear translocation, relatively few substances promote the nuclear translocation of SMAD proteins. These include importin 7 and importin 8 (Imp7 and Imp8) and the mammalian orthologues of Mask, which enhance the TGF-β signaling pathway transmission by assisting the nuclear translocation of SMAD proteins [143].

Besides these substances that can regulate the nuclear translocation of SMAD proteins, many other substances regulate SMADs protein in various ways. Examples include hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs/Hgs) and SARA, both of which can promote TGF-β signaling by activating SMAD2 and SMAD3. Another example is endosome-associated FYVE-domain protein (Endofin), which promotes TGF-β signaling by promoting the binding of SMAD4 to SMAD2. One final example is CXXC-type zinc finger protein 5 (CXXC5), which associates with the SMAD2/3 inhibitor histone deacetylase HDAC1 and competes with HDAC1 to bind to SMAD2/3, thereby eliminating the inhibitory effect of HDAC1 on TGF-β signal transduction and ultimately promoting TGF-β signal transduction [144–147]. In addition, *miR-326*, SKI, or SNON (members of the protooncoprotein family) can negatively regulate TGF-β signal transduction by inhibiting SMAD2, SMAD3, or SMAD4 [148, 149]. Transmembrane prostate androgen-induced protein (TMEPAI) and its homologs C18 ORF1, and ERBB2/Her2 receptor-interacting protein (ERBIN), among others, can also inhibit signal transduction by competing with SARA for binding to SMAD2/3 [150–152]. Interestingly, some substances can have a dual effect on SMAD proteins, both inhibiting and activating them. As a member of the GTPase Rho family (Rac1), Rac1 can inhibit TGF-β induced growth inhibition by inhibiting SMAD3 and promoting SMAD2 to enhance TGF-β-induced cell migration [153]. Nevertheless, how Rac1 coordinates the regulation of SMAD2 and SMAD3 in different cells is unknown.

4. Therapeutic Targeting of TGF-β Signaling Pathway

In the previous part of this article, we divided the molecules and mechanisms that regulate the TGF-β signaling pathway into three perspectives: the ligand; the receptor; and the

downstream conduction media. Similarly, here, we summarize these three perspectives and divide the molecular compounds that target TGF-β tumor therapy into three categories: (1) tumor therapy targeting TGF-β ligand; (2) tumor therapy targeting TGF-β receptor; (3) tumor therapy targeting the downstream mediator of TGF-β (Table 1).

4.1. Tumor Therapy Targeting TGF-β Ligand. Antisense oligonucleotides (ASO) are short strands of deoxyribonucleotide analogs that can be hybridized with complementary mRNA to cause mRNA degradation or interfere with mRNA maturation, thereby downregulating the target gene expression [154]. AP12009 (trabedersen) is a TGF-β2-specific ASO. Trabedersen inhibits the proliferation and migration of pancreatic cancer cells and reverses the immunosuppressive effect mediated by TGF-β2, thereby exerting its antitumor activity in vivo [155]. Clinical I/II studies have confirmed that using AP12009 can prolong patient survival time with malignant glioma [156]. These results indicate that AP12009 can become an effective treatment for malignant tumors. AP11014 is a specific ASO for TGF-β1. Ongoing preclinical research is studying the efficacy of AP11014 in non-small-cell lung cancer, colorectal cancer, and prostate cancer [157].

TGF-β is expressed in most cells as the latent form (L-TGF-β). TGF-β must be activated to exert its cell proliferation and invasion functions, immune regulation, and angiogenesis. The combination with integrin can activate TGF-β [158]. Therefore, blocking integrin-mediated TGF-β activation has also become a new strategy to target TGF-β signaling. In breast cancer models, the use of 264RAD, an antibody that blocks integrin $\alpha v\beta 6$, prevents tumor growth effectively [159]. However, in a trial using the antibody EMD121974 (cilengitide), which selectively inhibits $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, to treat head and neck squamous cell carcinoma (stages I and II, NCT00705016), cilengitide did not improve the median survival time of patients compared with standard chemotherapy [160]. Similarly, a phase III clinical trial of glioblastoma found that adding cilengitide to temozolomide chemoradiotherapy did not improve the treatment effect [161].

A monoclonal antibody is an effective tool to inhibit TGF-β signal transduction, which exerts antitumor activity in various tumor models by blocking TGF-β binding to its receptor. In the 4T1 syngeneic mouse model of metastatic breast cancer, the treatment of mice with 1D11 can significantly inhibit breast cancer's lung metastasis, related to the salivary bone protein (Bsp) in the metastasis [162]. The same researcher found that 1D11 can also inhibit lung metastasis in a mouse model of metastatic breast cancer by increasing $CD8^+$ T cells [163]. Another monoclonal antibody, 2G7, also exhibited a similar effect on inhibiting breast cancer metastasis by increasing NK cells' activity [164]. GC1008 (fresolimumab) is a high-affinity human monoclonal antibody that can neutralize the three active forms of TGF-β (TGF-β1, TGF-β2, and TGF-β3). Phase I studies have shown that GC1008 has significant antitumor activity in patients with advanced malignant melanoma and renal cell carcinoma [165]. In patients with metastatic breast cancer, fresolimumab combined with radiotherapy can improve patient median survival, which may be related to the enhanced systemic immune response. XPA-42-068, XPA-42-681, and XPA-42-089 are all human monoclonal antibodies with a high affinity that can neutralize various TGF-β isoforms. In a xenograft model of pharyngeal carcinoma, these antibodies can inhibit tumor growth [166]. At present, the pan-neutralizing antibody NIS793, which can block the three isotypes of TGF-β, is being used in the phase I/Ib study of patients with advanced malignant tumors combined with PD-1 antibody (PDR001) (NCT02947165).

4.2. Tumor Therapy Targeting TGF-β Receptors. With the participation of TβR III, activated TGF-β binds to TβR II with high affinity to recruit TβR I to the TGF-β/TβR II complex, phosphorylate SMAD2 and SMAD3, and initiate the signal transduction pathway. The TGF-β receptor plays a vital role in this pathway. Therefore, research on TGF-β receptor kinase inhibitors has also become a hot spot. At present, many TβR I (ALK5) inhibitors have been developed, most of which target the kinase domain of TβR I, thereby affecting the TGF-β signal transduction pathway. In many preclinical experiments, TβR I inhibitors have shown significant antitumor activity. As a small molecule selective inhibitor of ALK-5, SB431542 inhibits TGF-β-induced cell proliferation and migration in human glioma cells. It inhibits myeloma growth by restoring the terminal osteogenesis cell differentiation in a myeloma mouse model [167, 168]. SB431542 also inhibits the vasculogenic mimicry (VM) formation in xenografts in mouse models of breast cancer and inhibits tumor growth [169]. This discovery provides a new strategy for breast cancer treatment. Other studies have shown that SB431542 blocks HCC cell proliferation mediated by TGF-β signaling in vivo and in vitro related to the decrease of KLF6 expression in HCC cells [170]. SB505124 is another TβR I inhibitor that inhibits the activation of fibroblasts induced by TGF-β, thereby preventing esophageal squamous cell carcinoma- (ESCC-) induced neoangiogenesis [171]. In the pancreatic ductal adenocarcinoma mouse model, SB505124 significantly reduces pancreatic cancer cell proliferation, tumor growth, and metastasis [172]. SD208 is an oral TβR I inhibitor. In mouse models of pancreatic cancer and melanoma, SD208 inhibits pancreatic adenocarcinoma progression and reduces the development of melanoma bone metastasis and osteolytic lesions [173, 174]. In SW-48 colon adenocarcinoma cells, SD208 can also significantly downregulate the expression of oncogene miR-135b and reduce the occurrence of colon tumors [175]. LY2109761 is a small-molecule inhibitor that has stable pharmacokinetic characteristics that can inhibit TβR I and TβR II dually. In the mouse colon cancer cell CT26, LY2109761 reduces TGF-β-mediated cell migration and invasion. In vivo experiments have found that LY2109761 can reduce colon cancer liver metastasis and prolong the survival period of mice [176]. Similarly, in animal models of pancreatic cancer, LY2109761 inhibits abdominal organ metastasis of pancreatic cancer, especially liver metastasis, and improves its mortality [177]. In HCC cells, LY2109761 prevents HCC cell migration and invasion by upregulating E-cadherin [178] and can also exert antitumor

Table 1: Summary of targeted TGF-β drugs.

Therapy	Target	Drug	Phase
Targeting TGF-β ligand	TGF-β2mRNA	AP12009 (trabedersen)	I/II/IIb
	TGF-β1mRNA	AP11014	Preclinical
	TGF-β1, β2, β3	1D11	Preclinical
		2G7	Preclinical
		XPA-42-068, XPA-42-681	Preclinical
		GC1008 (fresolimumab)	I/II
		NIS793	I/Ib
	TGF-β1, β2	XPA-42-089	Preclinical
	$\alpha v\beta$6 Integrins	264RAD	Preclinical
	$\alpha v\beta$3, $\alpha v\beta$5 Integrins	EMD121974 (cilengitide)	I/II/III
Targeting TGF-β receptor	TβR I	SB431542	Preclinical
		SB505124	Preclinical
		SD208	Preclinical
		LY2157299 (galunisertib)	I/II
		LY3200882	I
		EW-7203, EW-7195	Preclinical
		EW-7197	I/II
	TβR I/II	LY2109761	Preclinical
	TβR II	CJJ300	Preclinical
	Chimeric antibody-TGF-β traps	CTLA4-TβR II	Preclinical
		PDL1-TβR II (M7824)	I/Ib/II/III
Targeting the downstream mediator of TGF-β	Smads	Trx-SARA	Preclinical
	pSmad3	TAT-SNX9	Preclinical

activity by inhibiting HCC neoangiogenesis [179]. LY2109761 can also reduce the migration and invasion of glioblastoma cells and inhibit new vessel formation [180]. Galunisertib (LY2157299 monohydrate) is an oral small-molecule inhibitor (SMI) of TβR I kinase, which can block the conduction of the TGF-β/ALK5 signaling pathway by downregulating the level of SMAD2 phosphorylation [181]. Studies have found that galunisertib inhibits TGF-β1-mediated EMT and tumor cell migration, reverses TGF-β1-mediated $CD8^+$ T cell and NK cell immunosuppression, and exerts a potent antitumor effect in various tumor models (including MX1 human xenograft breast cancer model, Calu6 human xenograft lung cancer model, and 4T1 breast tumor model) [182]. Phase II clinical trials have shown that galunisertib, combined with gemcitabine, can prolong patient median survival with unresectable pancreatic cancer and good safety [183]. Similarly, in a clinical phase IB study conducted in Japan, galunisertib, along with sorafenib, showed good safety and tolerability for treating patients with unresectable hepatocellular carcinoma [184]. However, in the phase II trial of recurrent glioblastoma, compared with lomustine plus placebo, the combined treatment of nilotinib and lomustine did not significantly improve patient overall survival [185]. At present, the second-generation ALK5 inhibitor LY3200882 has been developed. Compared with the LY2157299 compound, LY3200882 is more specific and potent. However, it is still in phase I clinical trial for treating patients with solid tumors, and its safety needs to be further verified (NCT02937272).

Many new TβR I small-molecule inhibitors have been developed recently, such as EW-7203 and EW-7195. These small-molecule inhibitors reduce the phosphorylation level of SMAD2 in vivo effectively and inhibit SMAD signaling and EMT induced by TGF-β1. In a mouse model of xenograft breast cancer, both EW-7203 and EW-7195 inhibit breast cancer lung metastasis [186, 187]. The study has found that compared with treatment with tyrosine kinase inhibitor (TKI) alone, the combined use of EW-7197 and TKI can delay the recurrence of the disease significantly in chronic myeloid leukemia (CML) mice, increase their survival period, and eliminate CML leukemia-initiating cells effectively [188]. Phase I/II clinical trials of EW-7197 (vactosertib) combined with other drugs for treating malignant tumors are currently underway, including metastatic gastric cancer (VAC + paclitaxel), advanced NSCLC (VAC + durvalumab), metastatic colorectal cancer, and gastric cancer (VAC + pembrolizumab), and progressive glioma (VAC + imatinib) [11]. The use of TβRI inhibitors inhibits most TGF-β signal transduction, but these kinase inhibitors usually lack specificity. At present, researchers have developed a small-molecule inhibitor CJJ300 that targets TβR II, which disrupts the formation of TGF-β-TβR I-TβR II signaling complex to inhibit the phosphorylation of SMAD and EMT induced by TGF-β. It is a novel mechanism to inhibit TGF-β signaling [189].

A new type of immunotherapeutic strategy has recently been developed for targeted TGF-β signal transduction, the bifunctional antibody-ligand trap. The antibody trap combines an antibody targeting CTLA-4 or PD-L1 and then fuses with the extracellular domain sequence of TβR II to disable TGF-β in the tumor microenvironment. Compared with standard anti-CTLA4 monotherapy, anti-CTLA4-TβR II molecules show more robust antitumor activity in human melanoma mouse models [190]. M7824 is an anti-PD-L1/TβR II fusion protein. Preclinical studies have found that M7824 can inhibit the EMT induced by TGF-β and exert antitumor activity in various antitumor models [191]. Phase I studies have shown that M7824 has good antitumor activity

in patients with advanced solid tumors and has stable safety [192]. Several phase II/III trials are underway to evaluate the effect of M7824 for treating malignant tumors currently, such as metastatic colorectal cancer or advanced solid tumors with microsatellite instability, locally advanced or metastatic biliary tract cancer, and advanced non-small-cell lung cancer (NCT03436563; NCT03833661; NCT03631706).

4.3. Tumor Therapy Targeting TGF-β Downstream Transducers. As an important downstream mediator of the TGF-β signaling pathway, the SMAD protein plays an important role in TGF-β signaling. Therefore, interference with SMAD expression will also affect TGF-β signaling. The peptide aptamer is a good example. It is a small-molecule protein that can bind to protein targets [193]. According to research reports, in NMuMG mouse mammary epithelial cells expressing the peptide aptamer Trx-SARA, Trx-SARA binds explicitly to SMAD2 and SMAD3, reducing the level of SMAD2-SMAD4 and SMAD3-SMAD4 complexes while inhibiting TGF-β-induced EMT [194]. Recently, a small-molecule peptide TAT-SNX9 that can specifically target phosphorylated SMAD3 (pSMAD3) was discovered. In a mouse lung fibrosis model, TAT-SNX9 inhibits TGF-β-mediated fibers by targeting pSMAD3 [195].

5. Conclusions and Perspectives

The members of the TGF-β family are highly conserved cell signaling proteins with multiple functions. They play an irreplaceable role in human body homeostasis by regulating cell proliferation, movement, differentiation, and apoptosis. The role of TGF-β in tumorigenesis and development is complex and contradictory. In the early stage of cancer, TGF-β suppresses cancer by inducing cell cycle arrest and apoptosis. However, in the later stages of cancer, TGF-β turns into a tumor promoter, which induces EMT and angiogenesis and inhibits immune cell activity, thereby evading immune surveillance and promoting tumor growth and invasion. TGF-β signaling is under fine regulation in the body. Once abnormally activated or inactivated, it may break the body's homeostasis, cause dynamic imbalance, and further promote tumor occurrence and development. Although researchers are developing or have developed many tumoricidal agents targeting TGF-β, the ideal clinical application of TGF-β targeted therapeutic drugs in oncology has not been achieved. Is this related to the dual, contradictory role of TGF-β in the tumor? Might some TGF-β-targeted antagonists not only inhibit the tumor-promoting effect of TGF-β but also inhibit the tumor inhibitory effect of TGF-β. Or is it related to cell specificity and tissue characteristics? Different tissues, different cells, and different tumor microenvironments have different responses to different antagonists. Some factors activate TGF-β in a certain tissue but may not or even have a negative effect on TGF-β in other tissues. This may also be a new challenge for developing TGF-β targeted therapy drugs. In the future, how should we make full use of the anticancer effect of TGF-β? Should we avoid or even limit the role of TGF-β in promoting cancer? How to combine cell specificity and tissue specificity to restrict the use of TGF-β targeted drugs in different diseases? These questions require further exploration and discovery.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Yun Yang, Wen-Long Ye, Ruo-Nan Zhang, and Xiao-Shun He equally contributed to this work.

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Erratum

Erratum to "Punicalin Alleviates OGD/R-Triggered Cell Injury via TGF-β-Mediated Oxidative Stress and Cell Cycle in Neuroblastoma Cells SH-SY5Y"

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In the article titled "Punicalin Alleviates OGD/R-Triggered Cell Injury via TGF-β-Mediated Oxidative Stress and Cell Cycle in Neuroblastoma Cells SH-SY5Y" [1], the Author's Contributions section is missing. The corrected section appears as follows:

Authors' Contributions

Tiansong Yang, Qingyong Wang, Yuanyuan Qu, and Yan Liu contributed equally to this work.

The error was introduced during the production process of the article, and Hindawi apologies for this mistake.

References

[1] T. Yang, Q. Wang, Y. Qu et al., "Punicalin Alleviates OGD/R-Triggered Cell Injury via TGF-β-Mediated Oxidative Stress and Cell Cycle in Neuroblastoma Cells SH-SY5Y," *Evidence-Based Complementary and Alternative Medicine*, vol. 2021, Article ID 6671282, 11 pages, 2021.

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Research Article

Antitumor Effects of Baicalein and Its Mechanism via TGFβ Pathway in Cervical Cancer HeLa Cells

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Background. Due to dual-regulating carcinogenesis, the TGFβ pathway is an ideal and alternative tumor target. Natural flavonoids possess the similar structures to estrogen and could exert an important benefit to cervical cancer. The present study aimed to screen the inhibitor of TGFβ pathway from natural flavonoids and evaluate the function and mechanism of the TGFβ pathway inhibitor on cervical cancer. *Materials and Methods*. The cervical cancer HeLa cells were firstly treated with different flavonoids and probed by western blot for screening the inhibitor of TGFβ pathway. And then, the effect of the identified inhibitor on cell proliferation was studied by CCK-8 and clone formation assay. Then, RT-PCR and western blot assay were performed to evaluate the effect of identified inhibitor on mTOR/p70S6K pathway, and the cell migration and EMT pathway were also examined using scratching analysis and western blot assay. Finally, the role of TGFβ was assessed via the classic inhibitor of TGFβ/SMAD pathway. *Results*. Screening data by western blot assay showed that baicalein displayed the best inhibitor effect on TGFβ expression. CCK-8 and clone formation assay showed that baicalein inhibited the cell proliferation and clone cell number. RT-PCR and western bolt for probing mTOR, p70S6K, and 4EBP1 revealed that baicalein could suppress their expression and phosphorylation. The scratching analysis and western blot assay displayed that baicalein inhibited the cell migration and EMT progression in HeLa. The use of SB431542, a TGFβ inhibitor, revealed that TGFβ was crucial to baicalein-regulating cell proliferation and migration in HeLa cells. *Conclusion*. Baicalein, a medicine agent screened from natural flavonoids targeting TGFβ pathway, could suppress mTOR/p70S6K pathway-mediated cell proliferation and EMT pathway-related migration via TGFβ pathway in cervical cancer HeLa cells.

1. Introduction

Cervical cancer ranked the third most frequent carcinoma in women all over the world, and there are more than 500,000 increasing incidents year by year. It accounts for approximately 275 100 cancer-related deaths annually [1–3]. Although the incidences and deaths of cervical cancer in developed countries are decreasing greatly, it remains the leading cause of cancer-associated deaths in female from the undeveloped region [4, 5]. In China, the morbidity and mortality of cervical cancer is evidently increasing, and it has contributed to the third leading cause of cancer deaths [6]. Currently, many antitumor agents have been reported and applied to the treatment of cervical cancer [7, 8]. However, the effective medicine for cervical cancer remains a challenge in the clinic. Hence, it is an urgent need to identify novel and potential candidates for the treatment of cervical cancer.

Natural products have been used in the folk medicine around the world for thousands of years. Due to the historically validated benefits to health and the rich chemical ingredients, the natural products have been becoming the material sources of modern medicines [9]. According to statistics, there are approximately 65% of modern pharmaceutical agents approved by the American Food and Drug

Administration (FDA) deriving from the ingredients of natural products [10, 11]. Among these complex ingredients of natural products, flavonoid is one kind of molecule with the similar structure to estrogen playing an indispensable role in the female physiology [12, 13]. Emerging evidences show that flavonoids could exert many benefit effects on cervical cancer. Liu et al. reported that some dietary flavonoids could inhibit invasion of cervical cancer via epithelial-mesenchymal transition signaling [14]. In another study, icariin displayed the inhibition effect on the growth of human cervical cancer cells by targeting the mTOR/PI3K/AKT signaling pathway [15]. This information reveals that flavonoid could be the important source of potential agents to treat cervical cancer.

The transforming growth factor (TGFβ) signal pathway is considered as a dual-regulator of carcinogenesis [16, 17]. At the early stages of tumorigenesis, the TGFβ signal pathway serves as a tumor inhibitor through promoting the cell apoptosis of premalignant tissues. However, at the advanced stages of tumorigenesis, TGFβ signal pathway began to lose the tumor inhibitory function with the oncogenic mutations of tumor cells and following turning into the tumor promotor via moving the tumor cells to subject epithelial-mesenchymal transition (EMT) and caused the tumor cells metastasis. The paradoxical role of TGFβ signal pathway in the different stages of carcinogenesis provides an extremely potential chance for drug development [18, 19]. Several evidences have verified that there are many compounds, including SB-431242, SD208, LY2109761, and IN-1130, exerting the antitumor activity via the TGFβ signal pathway [18]. Therefore, the TGFβ signal pathway is an ideal and alternative target for the development of antitumor medicine.

Here, we aimed to identify the potential candidate molecules targeting the TGFβ signal pathway for the treatment to cervical cancer from the natural flavonoids and elucidate the mechanism of the candidate treating against cervical cancer. The present study would provide an alternative therapy to cervical cancer and enrich the application of flavonoids.

2. Materials and Methods

2.1. Cell Culture. The human cervical cancer HeLa cells were cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Excell Bio, China) in a humidified atmosphere of 5% CO_2 at 37°C.

2.2. Western Blot. When the HeLa cells reached the 80–90% confluence, they were seeded and cultured for 12 hours. At the 12th hour, 20 μM or 50 μM flavonoids (Chengdu Biopurify, China) were added into the well of cell cultured plates and cultured for another 12 hours. At the 24th hour, the cells were lysed with NP40 lysis buffer (Solarbio, China), and the total protein was prepared and boiled for degeneration. 30 μg total protein was loaded into the SDS-PAGE and run for 2 hours. After 2 hours, the protein samples in gels were transferred to the PVDF membrane (Millipore, USA), blocked with 5% skim milk, and then were incubated with the primary antibody of TGFβ, mTOR, p70S6K, 4EBP1, p-mTOR, p-p70S6K, p-4EBP1, E-cadherin, Snail, FAK, and p-FAK (Proteinteck, China) for immunoreaction, then washed, incubated with secondary antibody, and visualized using an enhanced chemiluminescence system (Proteinteck, China).

2.3. CCK-8 Assay. When the HeLa cells reached the 80–90% confluence, they were seeded and cultured for 12 hours. At the 12th hour, the cells were added with 20 μM or 50 μM baicalein and cultured for six time of 12, 24, 36, 48, 60, and 72 hours. At the indicated time, CCK-8 solutions (Beyotime, China) were added into the well of cell cultured plates and cultured for another 1 hour. The absorbance of cells at 450 nm was detected using microplate reader (Bio-Tek, USA). The proliferation ratio was calculated as

$$\text{proliferation ratio}\,(\%) = \left(\frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}}\right) \times 100\%. \quad (1)$$

2.4. Clone Formation. When the HeLa cells reached the 70–80% confluence, they were seeded and cultured for 12 hours. At the 12th hour, the cells were added with 20 μM or 50 μM baicalein and cultured for one week. After one week, the cells were washed with PBS for twice, fixed with 4% paraformaldehyde for 5 minutes, stained with Gimsa (Shinoda, China) for 20 minutes, and photos were taken using camera.

2.5. RT-PCR. When the HeLa cells reached the 80–90% confluence, they were seeded and cultured for 12 hours. At the 12th hour, the cells were added with 20 μM or 50 μM baicalein and cultured for another 12 hours. At the 24th hour, the cells were lysed with trizol (Thermo, USA), and total RNA was extracted. The extracted RNA was reversely transcribed into the first-strand cDNA (Tiangen, China), and then PCR amplification of target genes of mTOR, p70S6K, and 4EBP1 was carried out in the PCR amplifier (Bio-Rad, USA). The amplification products were detected via gel electrophoresis. The primer sequences of mTOR, p70S6K, and 4EBP1 are as follows [20]:

mTOR: F: GCCGCAUUGUCUCUAUCAATT; R: UUGAUAGAGACAAUGCGGCTT

p70S6K: F: ACTTCTGGCTCGAAAGGTGG; R: TTGAGTCATCTGGGCTGTCG

4EBP1: F: CCTTTCCGGGACTTTCGCTTT; R: GCAGAATCCAGGTGGCAACA

2.6. Scratch Test. When the HeLa cells reached the 80–90% confluence, they were seeded into the 6-wells cell cultured plates which were premarked in the bottom of plated with the line as a reference for image acquisition. After 12 hours, the seeded cells were scratched with 10 μL pipette tip to form

a cell-free area and treated with 20 μM or 50 μM baicalein for another 24 hours. At the coming of the schedule time, the cells were imaged using microscope (Olympus, Japan).

2.7. Statistics Analysis. The statistical analysis was carried out using SPSS software (IBM, USA). The measurement data were presented as the mean ± SD. Comparisons between two groups were performed by the Student's t-test. The level of $p < 0.05$ was considered to be statically differences.

3. Results

3.1. Identification of TGFβ Inhibitor. To screen the inhibitor of TGFβ pathway, the expression of TGFβ in human cervical carcinoma HeLa cells exposed to the series of natural flavonoids were probed by western blotting assay. As displayed in Figure 1, there were five flavonoids weakly inducing the TGFβ expression and three flavonoids inhibiting the TGFβ expression (Figure 1(a)). Among the inhibitors, flavonoids 3 of baicalein exhibited the most evident inhibitory effect on TGFβ expression level (Figure 1(b), $^{**}p < 0.01$ vs. control group) and SMAD2/3 phosphorylation (Supplementary Figure S1). Its structure is shown in Figure 1(c). The result demonstrated that baicalein might be a potential inhibitor of TGFβ pathway and would become the object for the following study in the present research.

3.2. Effect of Baicalein on Cell Proliferation in HeLa Cells. The unlimited proliferation is the symbol events of tumor growth. To confirm the effect of TGFβ pathway inhibitor of baicalein on cervical cancer, CCK-8 and clone formation assay were used to examine the cell proliferation in human cervical cancer HeLa cells. The proliferation curve in Figure 1(a) showed that the proliferation ratio of cells exposed to baicalein displayed a time-dependent decrease from 0 to 72 hours. At the 72nd hour, the proliferation ratio of 20 μM and 50 μM baicalein was, respectively, 72.1% ± 5.1% and 45.7% ± 2.5%, demonstrating a concentration-dependent effect. Furthermore, the images of clone formation showed that the relative cell number of baicalein at 20 and 50 μM was significantly inhibited (Figure 2(b) and 2(c), $^{**}p < 0.01$ for 20 μM baicalein and $^{***}p < 0.001$ for 50 μM vs. control group). The similar data were observed in SKG IIIa cells (Supplementary Figure S2). These results demonstrated that baicalein could suppress the cell proliferation in HeLa cells.

3.3. Effect of Baicalein on mRNA Expression of mTOR, p70S6K, and 4EBP1 in HeLa Cells. The mTOR/p70S6K signal pathway is well known to regulate cell proliferation. To evaluate the relationship of mTOR/p70S6K pathway with the antiproliferation effect of baicalein on cervical cancer, the mRNA level of mTOR, p70S6K, and 4EBP1 was analyzed with RT-PCR. As seen in Figure 3, baicalein inhibited the mRNA expression of mTOR (Figure 3(a)), p70S6K (Figure 3(b)), and 4EBP1 (Figure 3(c)) at different degrees in HeLa cells, and the high concentration of 50 μM baicalein displayed all statistical differences compared with that in control group ($^{**}p < 0.01$ for mTOR, $^{*}p < 0.05$ for p70S6K, and $^{***}p < 0.001$ for 4EBP1). The results indicated that baicalein might suppress the mTOR/p70S6K signal pathway in HeLa cells.

3.4. Effect of Baicalein on Protein Expression and Phosphorylation of mTOR, p70S6K, and 4EBP1 in HeLa Cells. To further confirm the relationship of mTOR/p70S6K pathway with the antiproliferation effect of baicalein, western blot was employed to probe the protein expression and phosphorylation of mTOR, p70S6K and 4EBP1 in HeLa. As shown in Figure 4, both of 20 μM and 50 μM baicalein evidently inhibited the phosphorylation of mTOR ($^{**}p < 0.01$ for 20 μM and 50 μM baicalein vs. control group), 50 μM baicalein weakly inhibited the phosphorylation of p70S6K but no statistical differences, and 50 μM baicalein evidently inhibit the phosphorylation of 4EBP1. Meanwhile, 50 μM baicalein displayed a litter inhibitory effect on the expression of the three proteins. The similar effect by baicalein was observed in SKG IIIa cells (Supplementary Figure S3). Combined with the RT-PCR data, the results revealed that mTOR/p70S6K signal pathway may be in part contributed to the antiproliferation effect of baicalein in HeLa cells.

3.5. Effect of Baicalein on Cell Migration in HeLa Cells. Tumor cells migration is considered as one of the leading deaths causes of advanced carcinoma. To study the effect of baicalein on cervical cancer metastasis, scratch test was carried out to assess the cell migration ability. The images of HeLa cells displayed that, after the treatment with baicalein for 24 hours, the width of cells-free area is wider than that in the control group (Figure 5(a)). The statistical analysis showed that baicalein evidently inhibited the cell migration of HeLa cells ($^{*}p < 0.05$ for 20 μM and $^{**}p < 0.01$ for 50 μM baicalein vs. control group. The results demonstrated that baicalein could suppress the HeLa cells migration.

3.6. Effect of Baicalein on EMT Pathway in HeLa Cells. EMT pathway is an essential step of tumor metastasis from primary location to target location. To clarify the effect of baicalein on EMT pathway, western blot was employed again to probe the expression of E-cadherin, Snail, and FAK and the phosphorylation of FAK. As seen in Figure 6, 50 μM baicalein induced the expression of E-cadherin (Figure 6(a), $^{**}p < 0.01$ vs. control group) and inhibited the expression of Snail (Figure 6(b), $^{**}p < 0.01$ vs. control group) and the phosphorylation of FAK (Figure 6(c), $^{*}p < 0.05$ vs. control group); meanwhile, there were no changes in the three indicator of cells subjecting low concentration of 20 μM baicalein. These results demonstrated that baicalein could suppress the EMT pathway in cervical cancer HeLa cells.

3.7. The Role of TGFβ in Baicalein Suppressing Cervical Cancer in HeLa Cells. In order to verify the role of TGFβ in baicalein suppressing cervical cancer, SB431542, a TGFβ inhibitor,

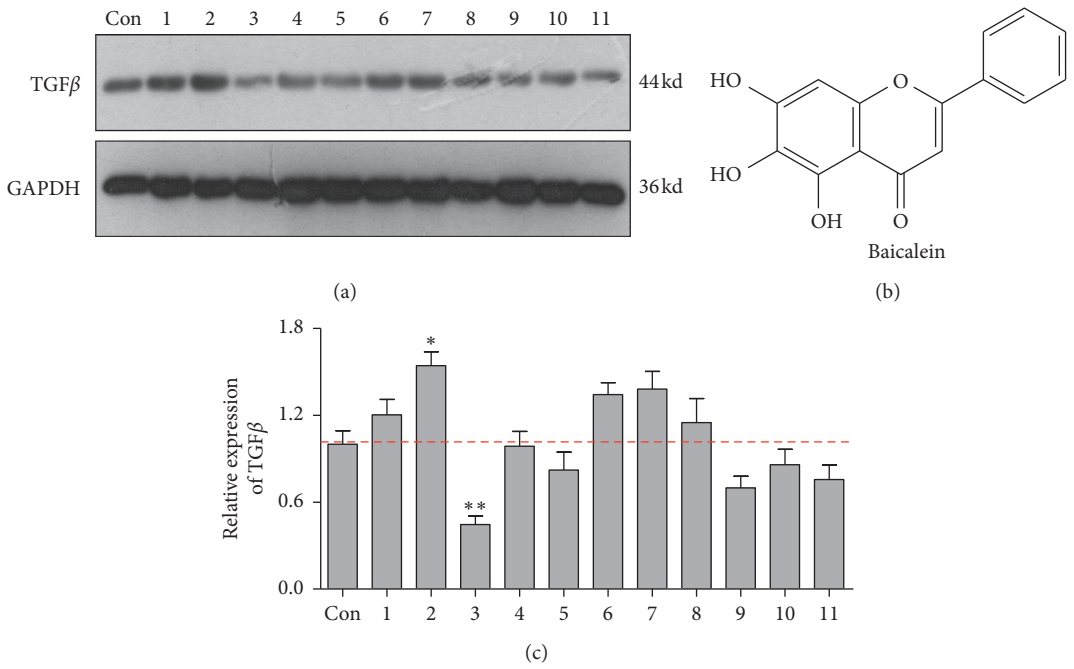

Figure 1: Baicalein is an inhibitor of TGFβ pathway. (a) Western blot assay for probing TGFβ expression. (b) The quantitative analysis of TGFβ expression. (c) The structure of baicalein. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group.

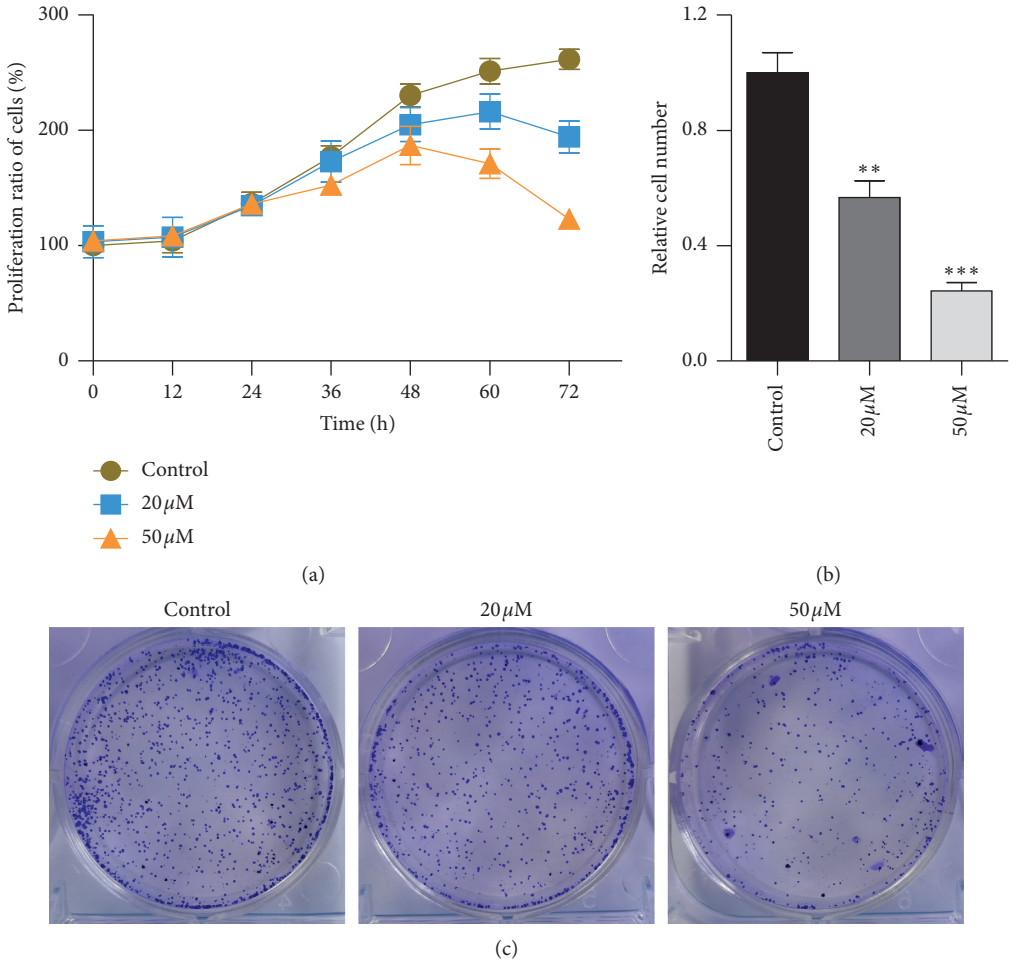

Figure 2: Baicalein suppresses the cell proliferation in HeLa cells. (a) CCK-8 assay for the curve of cell proliferation. (b) Colon formation assay for the cell proliferation. (c) The quantitative analysis of relative clone cell number. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group.

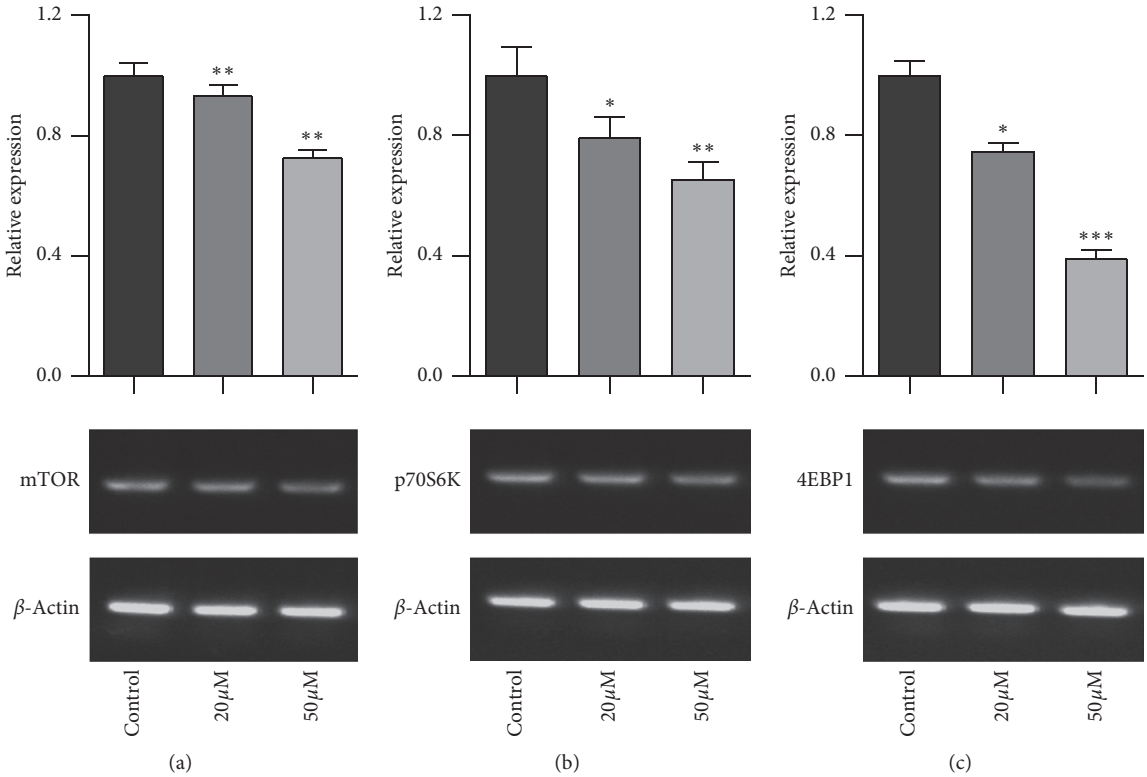

Figure 3: Baicalein inhibits the mRNA expression of mTOR (a), p70S6K, (b) and 4EBP1 (c). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control group.

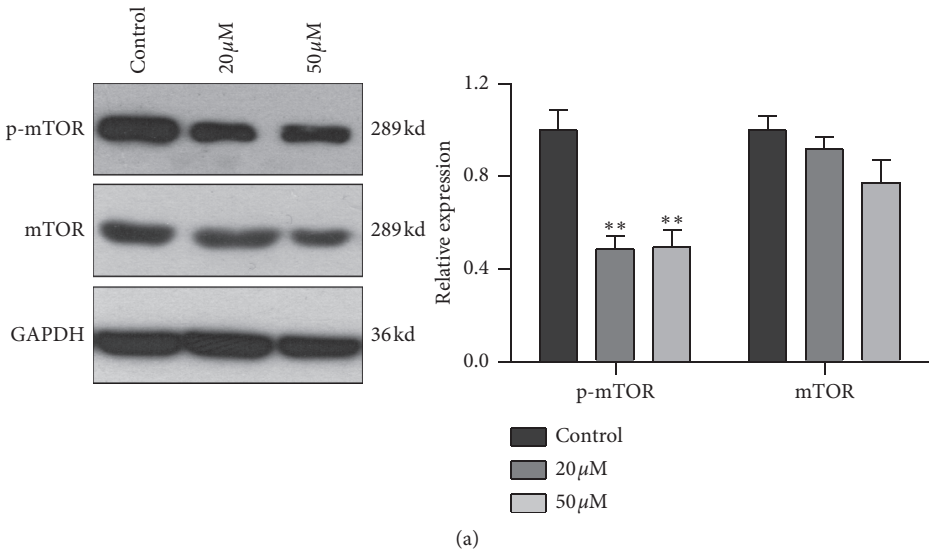

Figure 4: Continued.

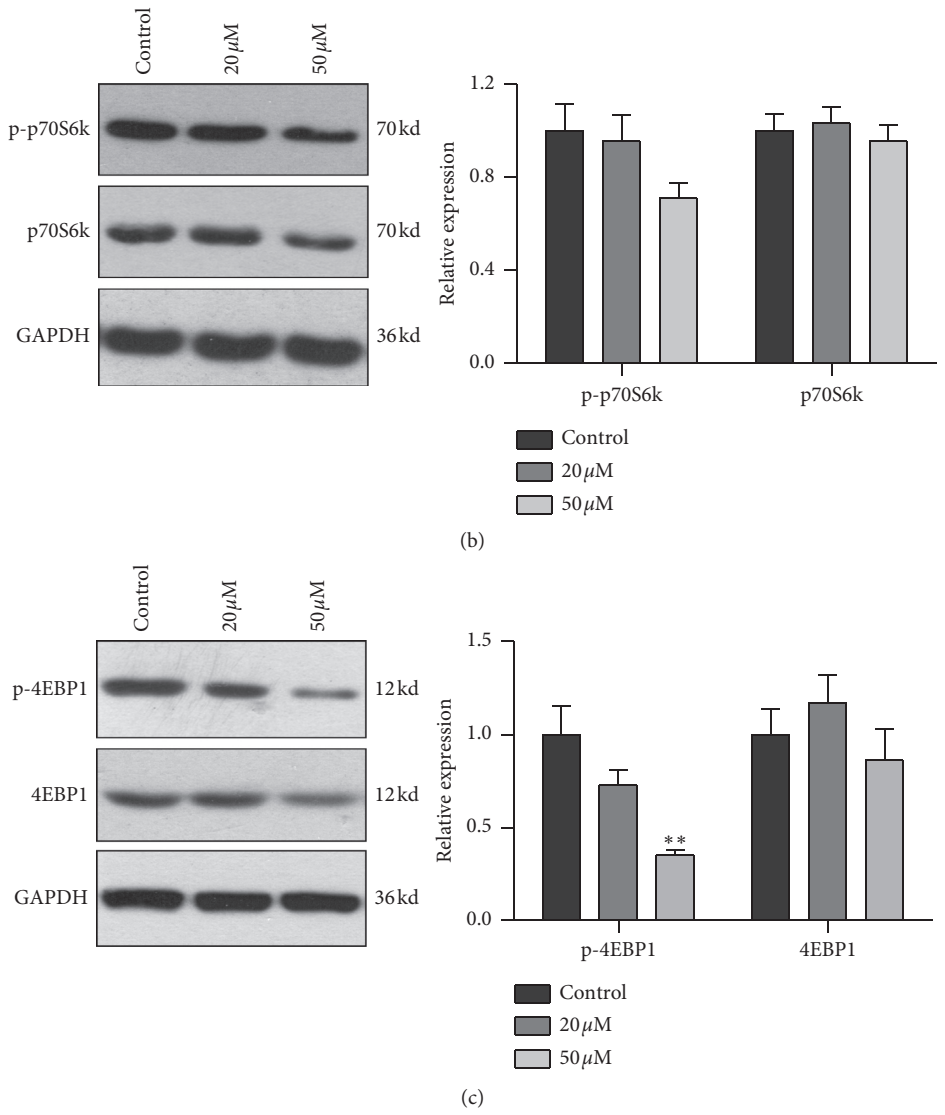

Figure 4: Baicalein inhibits the phosphorylation level of mTOR (a), p70S6K, (b) and 4EBP1 (c). $^{**}p < 0.01$ vs. control group.

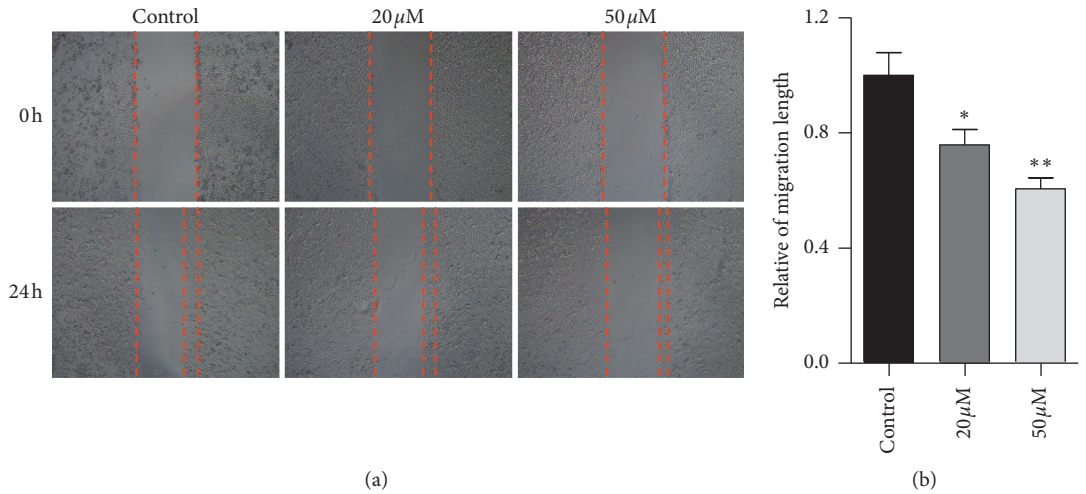

Figure 5: Baicalein suppresses the cell migration in HeLa cells. (a) Scratch assay for examining the cell migration. (b) The quantitative analysis for the relative migration length. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group.

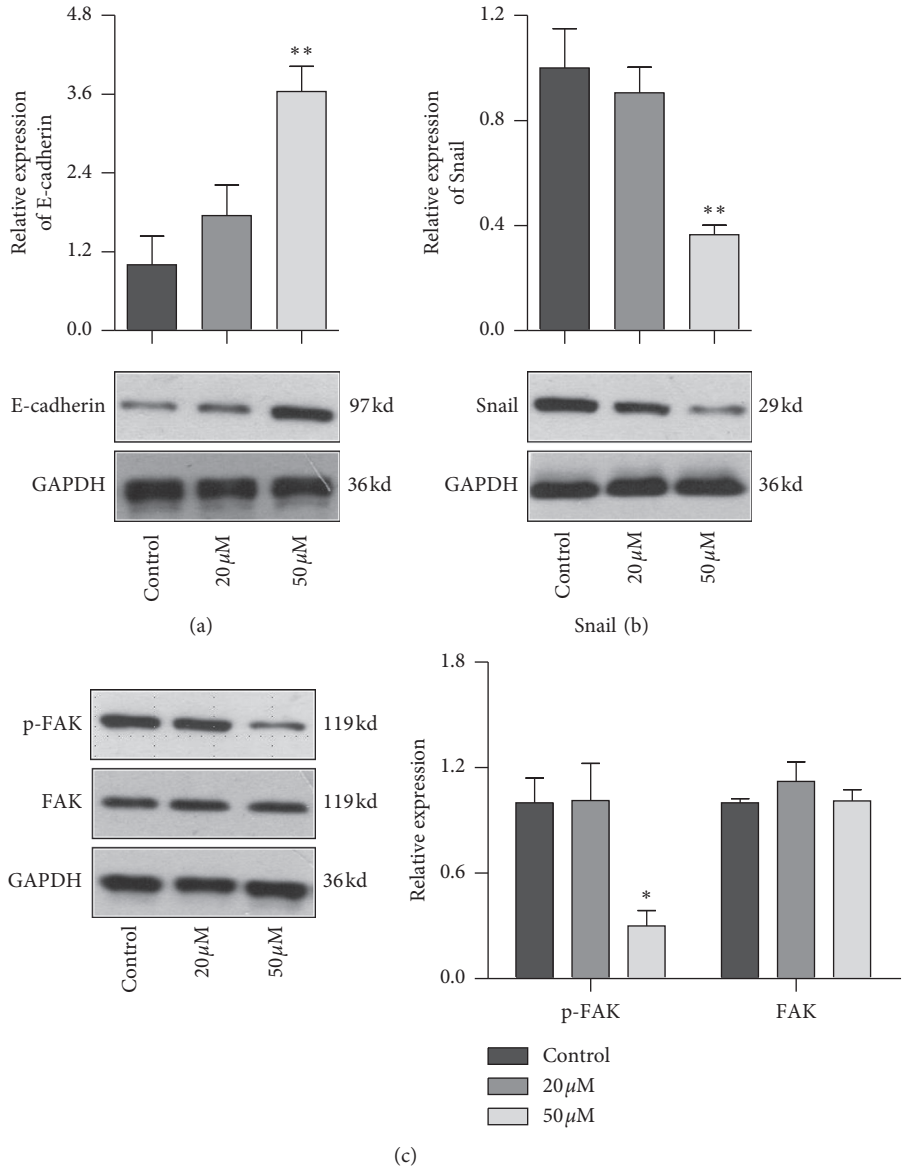

Figure 6: Baicalein suppresses the EMT pathway in HeLa cells. Western blot for probing the expression of E-cadherin (a), Snail (b), and FAK and p-FAK (c). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group.

was employed to further assess the effect of baicalein on the expression of p-mTOR, mTOR, and E-cadherin. As seen in Figure 7, the administration of only baicalein inhibited the phosphorylation of mTOR ($^{*}p < 0.05$ vs. control group) and induced the expression of E-cadherin ($^{**}p < 0.01$ vs. control group); however, the change trends were disappeared following the coadministration of baicalein and SB431542. These results demonstrated that TGFβ could be crucial to baicalein-mediating cell proliferation and migration.

4. Discussion

Flavonoid is recognized as a kind of natural molecules widely existing in the plates, which has the similar chemical structure with estrogen. The particular structures contribute to various biofunctions in gynecological disease including cervical cancer and breast cancer. Several flavonoids are reported to exhibit the antitumor effect on cervical cancer cells via suppressing the cell proliferation, metastasis, and invasion [15, 21, 22]. In addition, the TGFβ signal pathway is well known to promote the carcinogenesis via inducing the EMT pathway and angiogenesis at the advanced stages of tumor [23, 24]. Many compounds could target TGFβ signal pathway to suppress the cervical cancer. In this study, the inhibitor of the TGFβ signal pathway was screened from the natural flavonoids using western blot. We found that baicalein exerted the best inhibitory effect on TGFβ expression. The proliferation and migration tests revealed that baicalein could suppress the proliferation and migration of cells in cervical carcinoma HeLa cells. Additionally, the application of SB431542 revealed that the combination of baicalein and SB431542 reversed the effect of only baicalein on cell

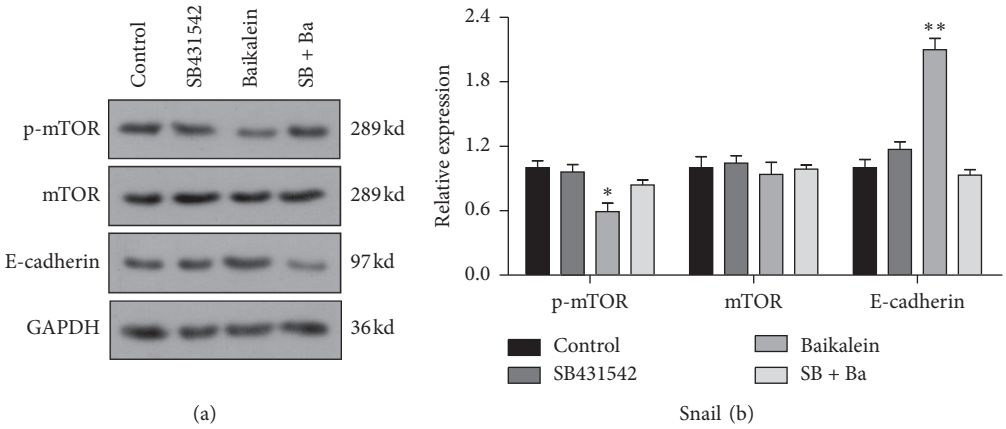

Figure 7: TGFβ is crucial to baicalein-mediating proliferation and migration in HeLa cells. Western blot for probing the expression of p-mTOR, mTOR, and E-cadherin. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group.

proliferation and migration in HeLa cells. These results indicated that the TGFβ signal pathway might involve in the effect of baicalein against cervical cancer.

Baicalein is a simple-structural flavonoid isolated from traditional Chinese herb of *Scutellaria baicalensis* which was applied to clear away heat and detoxicate in Chinese folk medicine for thousands of years [25, 26]. Inheriting the function of *Scutellaria baicalensis*, baicalein possesses various pharmacological activities such as eliminating inflammation, killing virus, and suppressing oxidant, historically. Many current investigations have also found that baicalein could suppress the different kinds of tumors via the proliferation and apoptosis. Yan et al. revealed that baicalein promoted autophagy and apoptosis of breast cancer cells through the PI3K/AKT pathway [27]. The study by Yu et al. showed that baicalein raised the cisplatin sensitivity to lung adenocarcinoma cells via the PI3K/Akt/NF-κB pathway [28]. It had been reported that baicalein could induce the apoptosis of colon cancer cells through inducing DEPP/Gadd45a and activating MAPKs [29]. The results in this study revealed the inhibitory effect of baicalein on proliferation and migration of cervical cancer HeLa cells via TGFβ signal pathway. Our findings make an expanding understanding for antitumor effects of baicalein and provide a valuable choice for the treatment to cervical cancer.

The unlimited proliferation is considered as one of the most typical characteristics of malignant tumor. It causes the extreme overgrowth of tumor tissue and predatory behavior to the nutrition and living space of normal tissue, eventually threatening to individual's life. The mTOR/p70S6K signal pathway plays an important role in the regulation of cell proliferation, which involves in the pathological process of cancer, diabetes, cardiovascular disease, and so on. In the tumorigenesis, mTOR protein is activated by the phosphorylation and then phosphorylated its substrate of p70S6K and 4EBP1, leading to the disordered regulation of cell cycle and uncontrolled cell proliferation. Several agents have been identified as an mTOR inhibitor to suppress the tumor [30, 31]. In this study, RT-PCR and western bolt assay displayed that baicalein inhibited the expression and phosphorylation of mTOR, p70S6K, and 4EBP1. Combined with the inhibitory effect of baicalein on cervical cancer HeLa cells proliferation, it is inferred that the mTOR/p70S6K signal pathway might involve in the inhibitory effect of baicalein on proliferation of cervical cancer cells.

Tumor metastasis is another important typical characteristic of malignant tumor, which is considered as the leading death cause of the advance-staged cancer patients. EMT pathway is an essential step of tumor metastasis from primary location to target location [32]. E-cadherin, Snail, and FAK are the three indicators of protein reflecting EMT pathway progression. Among these indicators, E-cadherin, an important member of calcium-dependent transmembrane glycoproteins, maintains the epithelial phenotype. Its decreased expression would trigger the falling of cell's adhesive ability and improvement of migration ability, promoting the EMT progression. Snail, a DNA-binding protein with zinc finger domains, could recognize and bind to the E-box in the promotor of E-cadherin and then inhibit the expression E-cadherin. FAK is a joint molecule gathering the signal protein to the focal adhesion, and its activation would also suppress the expression of E-cadherin. In the study, western blot data showed that baicalein initiated the induction of E-cadherin expression and the inhibition of Snail and p-FAK. These results indicated that baicalein could suppress the migration of cervical cancer HeLa cells via EMT pathway.

5. Conclusions

In summary, the present study found that baicalein is an inhibitor of TGFβ pathway, and it could suppress mTOR/p70S6K pathway-mediated cell proliferation and EMT pathway-related cell migration via TGFβ pathway in cervical cancer HeLa cells. In this context, we propose a potential mechanism of baicalein suppressing cervical cancer, which lays a theoretical basis for the development of baicalein and the treatment of cervical cancer.

Data Availability

The data during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Gang Yu and Lizhen Chen contributed equally to this work.

Acknowledgments

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Supplementary Materials

Figure S1: baicalein inhibits the phosphorylation of SAMD2 (a) and SMAD3 (b) in HeLa cells. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group. Figure S2: baicalein suppresses the cell proliferation in SKG IIIa cells via CCK-8 assay. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group. Figure S3: baicalein inhibits the phosphorylation level of mTOR (a) and p70S6K (b) in SKG IIIa cells. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group. . (*Supplementary Materials*)

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Research Article

Punicalin Alleviates OGD/R-Triggered Cell Injury via TGF-β-Mediated Oxidative Stress and Cell Cycle in Neuroblastoma Cells SH-SY5Y

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Purpose. The research aimed to identify the active component from *Punica granatum* L. to alleviate ischemia/reperfusion injury and clarify the underlying mechanism of the active component alleviating ischemia/reperfusion injury. *Materials and Methods*. The SH-SY5Y cell model of oxygen-glucose deprivation/reoxygenation (OGD/R) was established to simulate the ischemia/reperfusion injury. According to the strategy of bioassay-guided isolation, the active component of punicalin from *Punica granatum* L. was identified. Flow cytometry and Western blotting were employed to evaluate the effects of OGD/R and/or punicalin on cell cycle arrest. Immunofluorescence assay was applied to assess the nucleus translocation. The relative content of ROS and GSH and the enzyme activities of CAT and SOD were examined using ELISA. *Results*. The data of bioassay-guided isolation showed that punicalin from *Punica granatum* L. could alleviate OGD/R-induced cell injury in SH-SY5Y cells. Flow cytometry analysis and Western blotting for probing the expression of CDK1, p-CDK1, cyclin B1, and p21 revealed that punicalin could relieve OGD/R-induced cell cycle G0/G1 arrest. Additionally, immunofluorescence assay and Western blotting for probing the expression of TGF-β and p-Smad2/p-Smad3 showed that punicalin could relieve the OGD/R-induced TGF-β/Smad pathway. Furthermore, the TGF-β/Smad pathway inhibitor of LY2157299 was employed to confirm that the TGF-β/Smad pathway is crucial to the effect of punicalin. At last, it was indicated that punicalin could relieve OGD/R-induced oxidative stress. *Conclusion*. Punicalin, an active component from *Punica granatum* L., was identified as a protective agent to alleviate the OGD/R-induced cell injury, which could exert the protective effect via TGF-β/Smad pathway-regulated oxidative stress and cell cycle arrest in SH-SY5Y cells.

1. Introduction

Cerebral ischemic stroke is considered as a brain injury disease with the symptoms of hemiplegia, cognitive impairment, and disability, which are caused by clogging blood vessel leading to insufficiency of cerebral blood supply [1]. It accounted for approximately three million individual deaths all over the world in 2015, and the mortality is still rising year by year, seriously threatening human life and safety. Thrombolysis is one of the main strategies to treat ischemic stroke, and recombinant tissue plasminogen activator (rtPA) as a thrombolysis agent is now usually employed to alleviate the ischemic stroke through dissolving intravascular thrombus [2, 3]. Although the use of rtPA provides a remission in ischemic symptoms, it also triggers more severe side effects of brain damage and neuronal disorder referred to as ischemia/reperfusion injury (IRI). Several shreds of evidence indicated that oxidative stress, apoptosis, cell cycle arrest, and calcium overload could be involved in IRI [4, 5]. However, the complex pathological mechanisms remain

elusive, which also restricted the development of IRI medicinal agent. Hence, it is urgent and significant to elucidate the IRI progress and identify the potential candidate for clinical treatment in IRI.

The TGF-β/Smad pathway is recognized as a process of signaling cascade response to the growth factor for maintaining the individual's dynamic balance of physiology and pathology. It plays many crucial roles in cell proliferation, apoptosis, oxidative stress, and inflammation, participating in a series of disease events such as cancer, diabetes, and cardiocerebrovascular disease [6, 7]. Recent shreds of evidence showed that TGF-β could serve as an injury-related cytokine involving brain injury and neurodegenerative diseases. It is reported by Abdel et al. that propolis could relieve cerebral injury via the regulation of the TGF-β/Smad pathway [8]. Another study showed that the TGF-β/Smad pathway involved in the cerebral IRI after isoflurane exposure [9]. These findings imply us that the TGF-β/Smad pathway could be one of the crucial parts in IRI progress. Therefore, identifying the TGF-β inhibitor would be a potential strategy to develop a medicinal agent for IRI treatment.

Punica granatum L. (PGL), also named pomegranate, has been wildly recognized as an edible fruit for hundreds of years in the earth. Recently, increasing shreds of evidence revealed that PGL has many bioactivities including anti-inflammation and antioxidation and exert a vast benefit in treatment and prevention of diseases such as diabetes, cancer, cardiovascular disease, and ischemia injury [10, 11]. The component analysis indicated that there are a series of active compounds containing procyanidin, corilagin, ellagic acid, and punicalin exiting in PGL. Among these, procyanidin reported to have a protective effect against brain damage in mice via alleviating cell apoptosis, oxidative stress, and induced angiogenesis [12, 13]. Corilagin possessed oxidation-inhibiting and angiogenic-evoking effects to ameliorate cerebral ischemia [14], and ellagic acid could relieve cerebral injury through mediating neuron apoptosis and restoring brain-blood barrier (BBB) [15]. The information as mentioned earlier demonstrated that PGL could be a valuable treasure chest of potential medicinal agents' development for the treatment of diseases especially IRI.

Here, we established an oxygen-glucose deprivation/reoxygenation (OGD/R) model in human neuroblastoma cell SH-SY5Y to simulate IRI, identify a TGF-β inhibitor of punicalin alleviating OGD/R-induced neuroblastoma injury from PGL extraction, and elucidate the mechanism of punicalin-suppressing cell injury via TGF-β-mediated oxidative stress and cell cycle arrest. The present study revealed the role of TGF-β in IRI development and treatment and provided a novel therapeutic approach for IRI.

2. Materials and Methods

2.1. Chemicals and Reagents. DMEM medium (Cat No. C11995) and FBS (Cat No.10099-141) were purchased from Gibco (USA). MTT reagent and propidium iodide (PI, Cat No. P8080) were obtained from Solarbio (China). The primary antibody of CDK1 (Cat No. ab133327), p-CDK1 (Cat No. ab201008), cyclin B1 (Cat No. ab32053), p21 (Cat No. ab109520), TGF-β (Cat No. ab215715), and Smad 2/Smad3 (Cat No. ab202445) and the secondary antibody were purchased from Abcam (USA). The dyes of DAPI (Cat No. 268298) were purchased from Sigma (USA). The ELISA assay kits of ROS (Cat No. E004-1-1), CAT (Cat No. A007-1-1), GSH (Cat No. A005-1-2), and SOD (Cat No. A001-3-2) were obtained from Nanjing Jiancheng Bioengineering Institute (China). The LY2157299 (Cat No. HY-13226), a TGF-β inhibitor, was purchased from MedChemExpress (MCE). The organic solvents ethanol, petroleum ether, chloroform, and ethyl acetate were purchased from Sinopharm Chemical Reagent Co., Ltd (China).

2.2. Preparation of Punica granatum L. Extraction. The fruit of *Punica granatum* L. was purchased from a farmers' market in Harbin city, and the seeds were taken out. The collected seeds were dried in a vacuum drying oven and ground to powder using crushing machinery. The powder (500 g) was refluxed and extracted with 2.5 L 60% ethanol for 2 hours, and the extraction was repeated thrice. The extraction solution was merged and evaporated in rotary evaporation to no alcohol taste. After that, the ethanol extraction was furthermore extracted with petroleum ether, chloroform, and ethyl acetate, respectively, to obtain three fractions of PE, CF, and EA. The three fractions were dried to solid in rotary evaporation, and the active compound was identified according to bioactivity-guided isolation.

2.3. Cell Culture. Human neuroblastoma SH-SY5Y cells were purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences, and Chinese Academy of Sciences. The SH-SY5Y cells were cultured in DMEM mediums with 10% FBS and the condition of 5% CO_2 at 37°C.

2.4. Construction of the OGD/R-Induced Cellular Injury Model. The SH-SY5Y cells were seeded into a 6-well plate and cultured in the DMEM medium with glucose-free. The culture conditions are 5% CO_2, 1% O_2, and 94% N_2 at 37°C for 6 hours. And then, the medium was replaced with normal DMEM medium and cultured in the normal atmosphere of 5% CO_2 for another 24 hours to reoxygenate.

2.5. MTT Assay. SH-SY5Y cells were seeded into the 96-well plate and cultured for 12 hours. And then, the seeded cells were treated with OGD/R and *Punica granatum* L./punicalin at the response concentration for the specific time. After treating, the cells were added with 20 μL MTT (5 mg/mL), cultured for another 3 hours, and diluted with 100 μL DMSO, and the OD value was read using a microplate reader (Peiqing, China). The proliferation rate was calculated using Microsoft Excel software as

$$\text{Proliferation rate} = \frac{\left(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}\right)}{\left(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}\right)} \times 100\%. \quad (1)$$

2.6. EdU Incorporation Assay. SH-SY5Y cells were seeded into a 12-well plate and cultured for 12 hours. And then, the seeded cells were treated with OGD/R and *Punica granatum* L./punicalin at the response concentration for the specific time. After treating, the cells were added with 300 μL EdU medium (50 μM), cultured for another 2 hours, and washed with PBS twice. The cell images were captured with a fluorescence microscope (Nikon, Japan).

2.7. Cell Cycle Distribution Analysis. SH-SY5Y cells were seeded into a 6-well plate and cultured for 12 hours. And then, the seeded cells were treated with OGD/R and punicalin for the specific time. After treating, the cells were digested into the state of signal cells, collected the cells into 1.5 mL tubes, fixed with precold 70% alcohol at room temperature for 30 min, and stained with PI for another 30 min. The stained cells were detected by flow cytometry (Beckman, USA).

2.8. Western Blotting. SH-SY5Y cells were seeded into a 6-well plate and cultured for 12 hours. And then, the seeded cells were treated with OGD/R and punicalin for the specific time. After treating, the cells were lysed using RIPA lysis buffer, centrifuged, and harvested the total protein. Total protein was subjected to the SDS-PAGE for separation, transferred protein to PVDF membrane, blocked PVDF membrane with 5% fat-free milk, incubated with primary antibody of CDK1, p-CDK1, cyclin B1, p21, TGF-β, and p-Smad2/Smad3 overnight at 4°C, washed with TBST for three times, incubated the response secondary antibody at room temperature for 1 hour, washed for three times, and at last probed with ECL reagents.

2.9. Immunofluorescence. SH-SY5Y cells were seeded to the slices in a 12-well plate and cultured for 12 hours. And then, the seeded cells were treated with OGD/R and punicalin for the specific time. After treating, the slices were fixed with formaldehyde, perforated with triton X-100 solution, fixed with BSA, incubated with primary antibody of Smad3 and secondary antibody at room temperature, and stained with DAPI and sealed. At last, the slices were examined using a confocal microscope.

2.10. Statistical Analysis. The data are expressed as the mean ± SD, and the analysis is performed with SPSS software (USA). Statistical significance is defined as $p < 0.05$.

3. Results

3.1. The Effects of Punica granatum L. Extraction on OGD/R-Induced Cellular Injury in SH-SY5Y Cells. The change of cellular morphology and proliferation rate is the appearance indicators to assess cell damage. Microscope photograph (Figure 1(a)) showed that the outline of SH-SY5Y cells subjecting to OGD/R treatment was dispersive and ambiguous, and the amount was declined compared with that of SH-SY5Y cells in the normal control group (NC). *Punica granatum* L. extraction at different concentrations of 1 mg/mL and 2 mg/mL ameliorated the cellular morphology change induced by OGD/R. The MTT assay (Figure 1(b)) revealed that the living cell amount subjecting to the supplement of 2 mg/mL *Punica granatum* L. extraction for 24, 48, and 72 hours was close to the amount in normal control groups and markedly more than that of OGD/R groups ($^{\#}p < 0.05$ for 48 hours and $^{\#\#}p < 0.01$ for 72 hours). DNA synthesis determines the cellular proliferation and amounts, indirectly reflecting the cell damage. Furthermore, the EdU incorporation assay was employed to evaluate DNA synthesis. As shown in Figure 1(c), the proportion of EdU incorporation in the OGD/R group was significantly suppressed compared to that in the normal control group ($^{***}p < 0.001$) in SH-SY5Y cells. *Punica granatum* L. extraction administration at different doses ameliorated the suppression induced by OGD/R, and 2 mg/mL *Punica granatum* L. extraction raised the EdU incorporation proportion from 0.46 to 0.80 ($^{\#\#}p < 0.01$). The results demonstrated that *Punica granatum* L. extraction could alleviate OGD/R-induced cellular injury in SH-SY5Y cells.

3.2. Identification of Punicalin as an Active Component from Punica granatum L. Extraction. The bioassay-guided isolation was employed to determine the active component with the potential of protection against OGD/R-induced cellular injury from *Punica granatum* L. The powdered sample was extracted with ethanol to obtain ethanol extract (EE). The resulted ethanol extract was fractionated petroleum ether (PE), chloroform (CF), and ethyl acetate (EA). The extraction route is shown in Figure 2(a). Then, the protective effect of the ethanol extraction and the three fractions against cell injury were evaluated by the proliferation test. As shown in MTT data of Figure 2(b), OGD/R suppressed the proliferation of human neuroblastoma SH-SY5Y cells ($^{***}p < 0.001$), and the ethanol extraction and EA fraction exhibited a particular alleviation of OGD/R-induced proliferation suppression ($^{\#}p < 0.05$). Next, the EA fraction was subjected to column chromatography on silica gel with the mobile phase of the chloroform-methanol solution and preparative high-performance liquid phase (pHPLC) with the mobile phase of methanol-water to obtain several compounds. The protection activities of the isolated compounds against cell injury were detected by the MTT assay (the data were not shown). The expected active compound was identified as punicalin, and the structure is shown in Figure 2(c).

3.3. Effect of Punicalin on OGD/R-Induced Cell Cycle Arrest. In order to better understand the role of punicalin in human neuroblastoma SH-SY5Y cells subjecting to OGD/R, cell cycle distribution and its marker proteins were tested, respectively, by flow cytometry and Western blotting. Flow cytometry (Figure 3(a)) revealed that the cells treated by OGD/R exhibited G0/G1 arrest and the ratio of G0/G1 phase rising from 24.7% to 37.4% compared to the normal control group. After the preexposure of punicalin at different concentrations, the ratio of the G0/G1 phase of 10 μM and 20 μM was, respectively, 37.6% and 28.9%, and a high dose of punicalin alleviated the rising of G0/G1 phase ratio in the

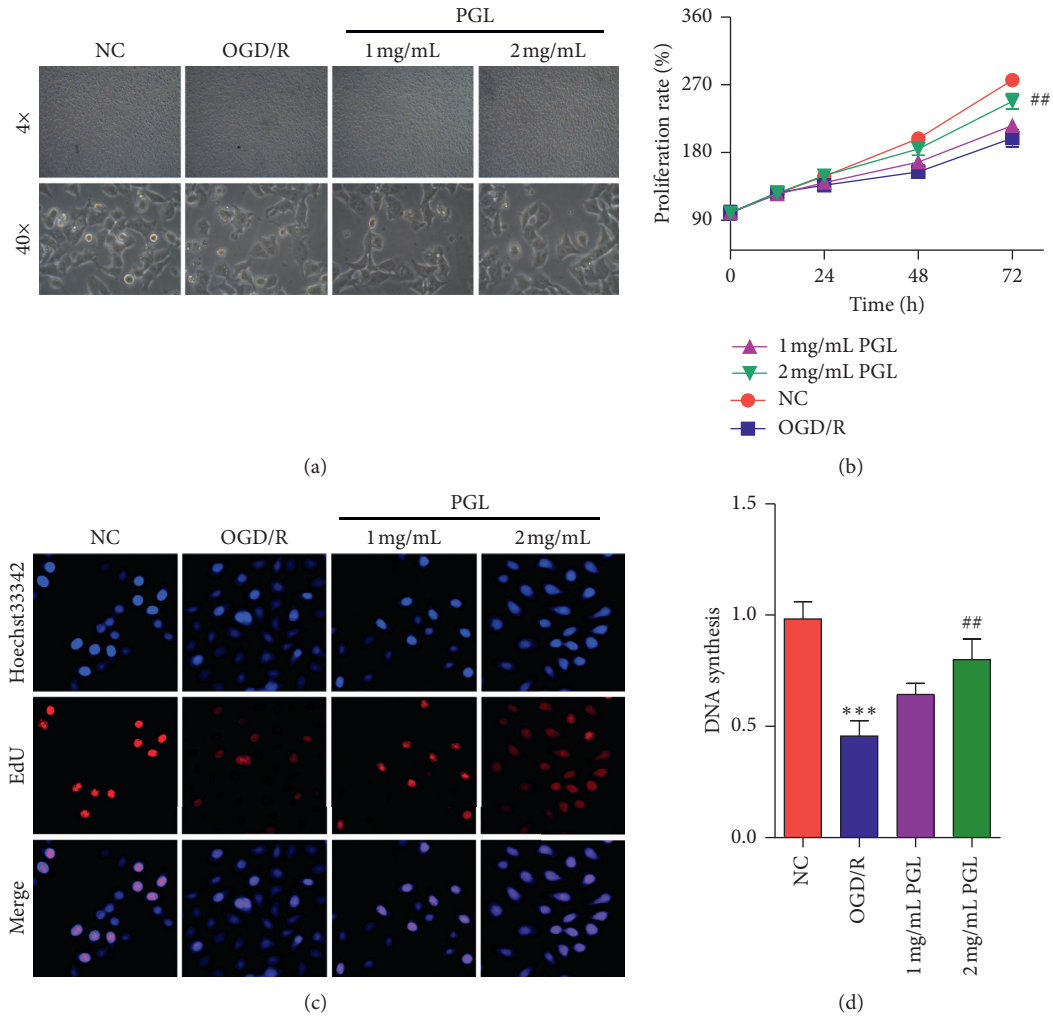

Figure 1: The extraction of *Punica granatum* L. alleviates OGD/R-induced cellular injury in SH-SY5Y cells. (a) The representative image of SH-SY5Y cells subjecting to OGD/R and/or *Punica granatum* L. extraction. (b) The proliferation rate of cells. (c) DNA synthesis of cells. PGL, *Punica granatum* L. $^{***}p < 0.001$ compared to the normal control, and $^{\#\#}p < 0.01$ compared to the OGD/R group.

OGD/R group. The protein marker expression reflecting G0/G1 phase progress (Figure 3(b)) indicated that OGD/R could suppress the expression of cyclin B1 and cyclin-dependent kinases CDK1 and induce the expression of p21 and phosphorylation of CDK1. Punicalin at 20 μM ameliorated OGD/R-mediated expression of cyclin B1, CDK1, p21 and phosphorylation of CDK1, which is consistent with the data of cell cycle arrest. These results demonstrated that punicalin could alleviate OGD/R-induced cell cycle G0/G1 arrest in SH-SY5Y cells.

3.4. Effect of Punicalin on the TGF-β Signal Pathway in SH-SY5Y Cells. The TGF-β signal pathway is usually activated by biological stimuli, causing the phosphorylation and nucleus localization of Smad2 and Smad3, following inducing related genes expression, and subsequently leading to the occurrence of physiopathology contain IRI. To evaluate the effect of the identified candidate punicalin on the TGF-β signal pathway, Western blotting and the immunofluorescence assay were used to examine the level of TGF-β, phosphorylation of Smad2 and Smad3, and nucleus localization of Smad3. Figure 4(a) indicates that OGD/R induced the TGF-β expression compared to the normal control group and 20 μM of punicalin inhibited the induction of TGF-β expression by OGD/R in SH-SY5Y cells. Similarly, the phosphorylation of Smad2 and Smad3 were induced by OGD/R, and a high dose of punicalin ameliorated the induction of phosphorylation. Furthermore, immunofluorescence image (Figure 4(b)) showed that OGD/R promoted the translocation of Smad3 from the cytoplasm to nucleus; meanwhile, punicalin at 20 μM concentration alleviated the nucleus translocation of Smad3 promoted by OGD/R. Taken together, OGD/R could activate the TGF-β signal pathway, and punicalin possesses the ability to attenuate the activation of the TGF-β pathway by OGD/R in SH-SY5Y cells.

3.5. The Role of the TGF-β Pathway in the Effect of Punicalin on OGD/R-Induced Cellular Injury. The abovementioned data demonstrated that punicalin could relieve OGD/R-induced neuroblastoma injury and the TGF-β signal pathway.

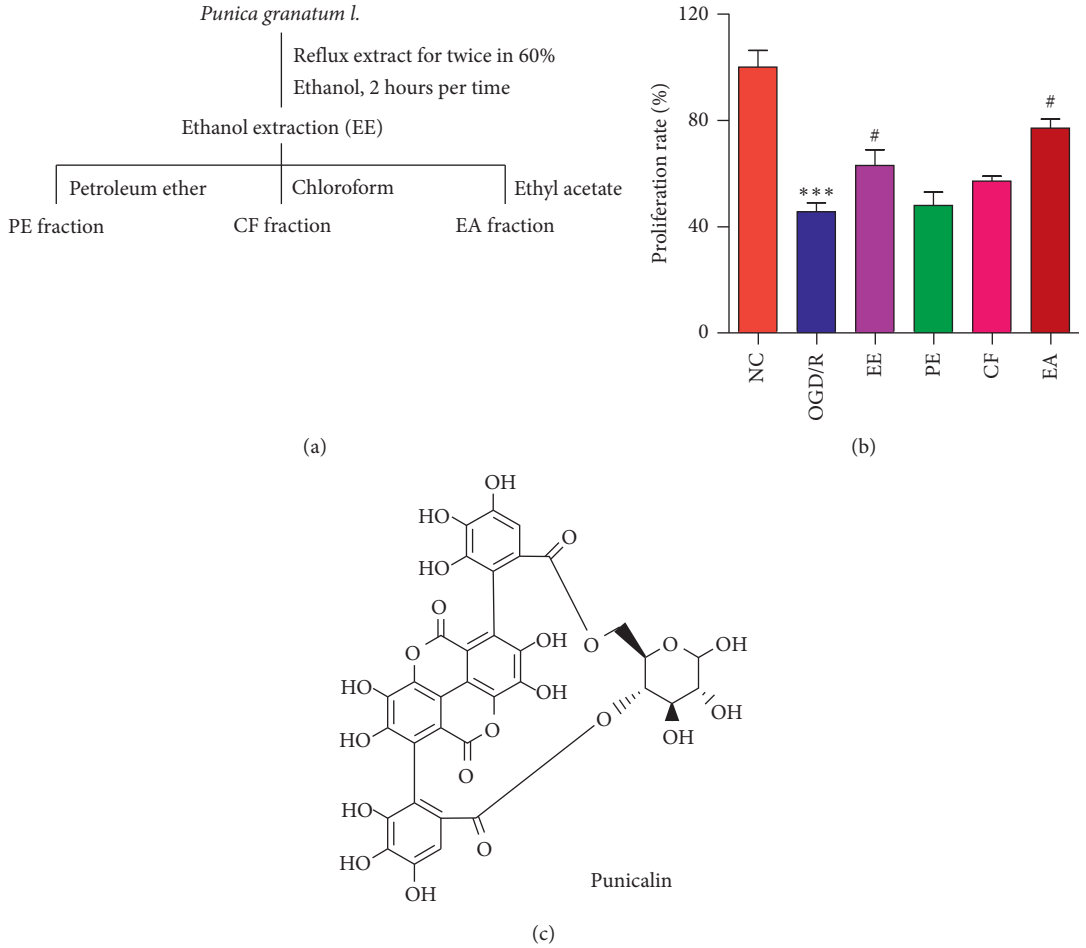

Figure 2: Identifying punicalin as an active component from *Punica granatum* L. (a) The separation process of *Punica granatum* L. (b) The proliferation rate of cells subjecting to the fractions of *Punica granatum* L. (c) The structure of punicalin. $^{***}p < 0.001$ compared to the normal control, and $^{\#}p < 0.05$ compared to the OGD/R group.

Combined with the shreds of evidence of TGF-β-mediating brain injury progress, it has speculated that the TGF-β pathway maybe involved in the effect of punicalin on OGD/R-induced neuroblastoma injury. To confirm the role of the TGF-β pathway in punicalin alleviating OGD/R-induced cellular injury, LY2157299 was employed as a tool agent inhibiting the TGF-β pathway in SH-SY5Y cells. As shown in line chart by the MTT assay (Figure 5(a)), the proliferation ratio in the punicalin group was higher than that in the OGD/R group, and the p value was, respectively, $^{\#}p < 0.05$ for 48 hours and $^{\#\#\#}p < 0.001$ for 72 hours. Combined supplement of punicalin and LY2157299 exhibited only a litter promotion to cellular proliferation compared to the OGD/R group and no significant difference even for 72 hours in SH-SY5Y cells. Furthermore, the EdU incorporation assay (Figure 5(b)) showed that punicalin significantly raised the DNA synthesis compared to the OGD/R group ($^{\#\#\#}p < 0.001$), and the combined supplement of punicalin and LY2157299 had no raised effect on DNA synthesis. These results demonstrated that the TGF-β pathway is involved in the effect of punicalin alleviating OGD/R-induced neuroblastoma injury in SH-SY5Y cells.

3.6. Effect of Punicalin on OGD/R-Induced Oxidative Stress. To furthermore study the effect of punicalin on oxidative stress during OGD/R-induced neuroblastoma injury, the indicators of ROS, CAT, GST, and SOD reflecting the oxidative stress level (Figure 6(a)) were detected using the ELISA assay. As seen in Figures 6(b)–6(e), OGD/R raised the relative content of ROS, suppressed the relative level of CAT and GSH, and reduced the enzyme activity of SOD compared to the normal control group. Expectedly, punicalin inhibited the induction of ROS content by OGD/R and alleviated the inhibition of CAT and GSH level and SOD enzyme activity. These data implied that punicalin could ameliorate OGD/R-induced oxidative stress in human neuroblastoma SH-SY5Y cells The scheme summarizing the mechanism of punicalin attenuating OGD/R-induced neuroblastoma injury is shown in Figure 7.

4. Discussion

Ischemia/reperfusion injury is considered as a brain and blood vessel complication trigged by the thrombolysis treatment to ischemic stroke, which has given a severe threat to human

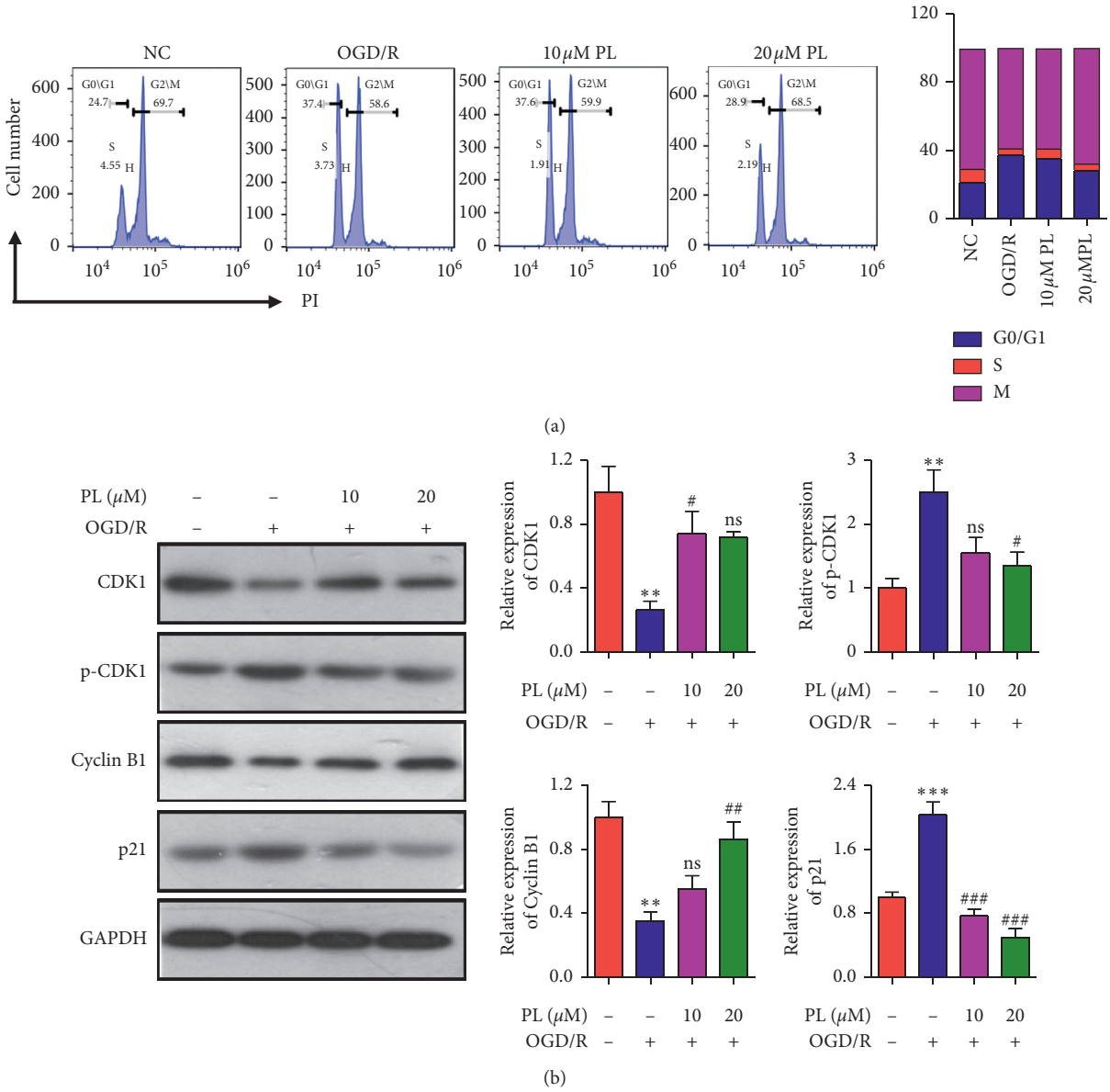

Figure 3: Punicalin alleviates OGD/R-induced cell cycle arrest in SH-SY5Y cells. (a) The cell cycle distribution of cells subjecting to OGD/R and/or punicalin. (b) The expression of cell cycle G1 phase-related proteins of CDK1, p-CDK1, cyclin B1, and p21. PL, punicalin. $^{**}p < 0.01$ compared to the normal control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared to the OGD/R group, and ns represents not significant.

health and happiness. Recent research studies have shown that several traditional Chinese medicines and edible plants, such as Qingkailing injection [16], Yiqi Tongluo granule [17], and *Crepidiastrum denticulatum* extract [18], could relieve ischemia/reperfusion injury. *Punica granatum* L. is an edible fruit with plenty of bioactivities for hundreds of years. It has been reported that *Punica granatum* L. could protect against the injury of the lung, kidney, and myocardium [10, 19]. However, the effect of *Punica granatum* L. extraction and its active components on ischemia/reperfusion injury remains rarely reported. In the current research, the OGD/R model in SH-SY5Y cells was established to simulate IRI. It was applied to screen the protective agents of IRI from *Punica granatum* L. with the strategy of bioassay-guided isolation. Interestingly, an active component of punicalin was found to inhibit OGD/R-induced cellular injury. The finding expands the nutritive value of *Punica granatum* L. and also provides a possible and alternative chance to the prevention and treatment to ischemia/reperfusion injury.

Punicalin is recognized as one member of tannins family, which exists explicitly in the husks, seeds, and leaves of *Punica granatum* L. The pharmacological studies show that punicalin possessed the function of resisting oxidation, protecting against liver damage and removing inflammation [20, 21]. It has been reported that punicalin could alleviate the acetaminophen or carbon tetrachloride-induced liver damage via its antioxidative and hepatoprotective activities [22, 23]. However, the effect of punicalin on ischemia/

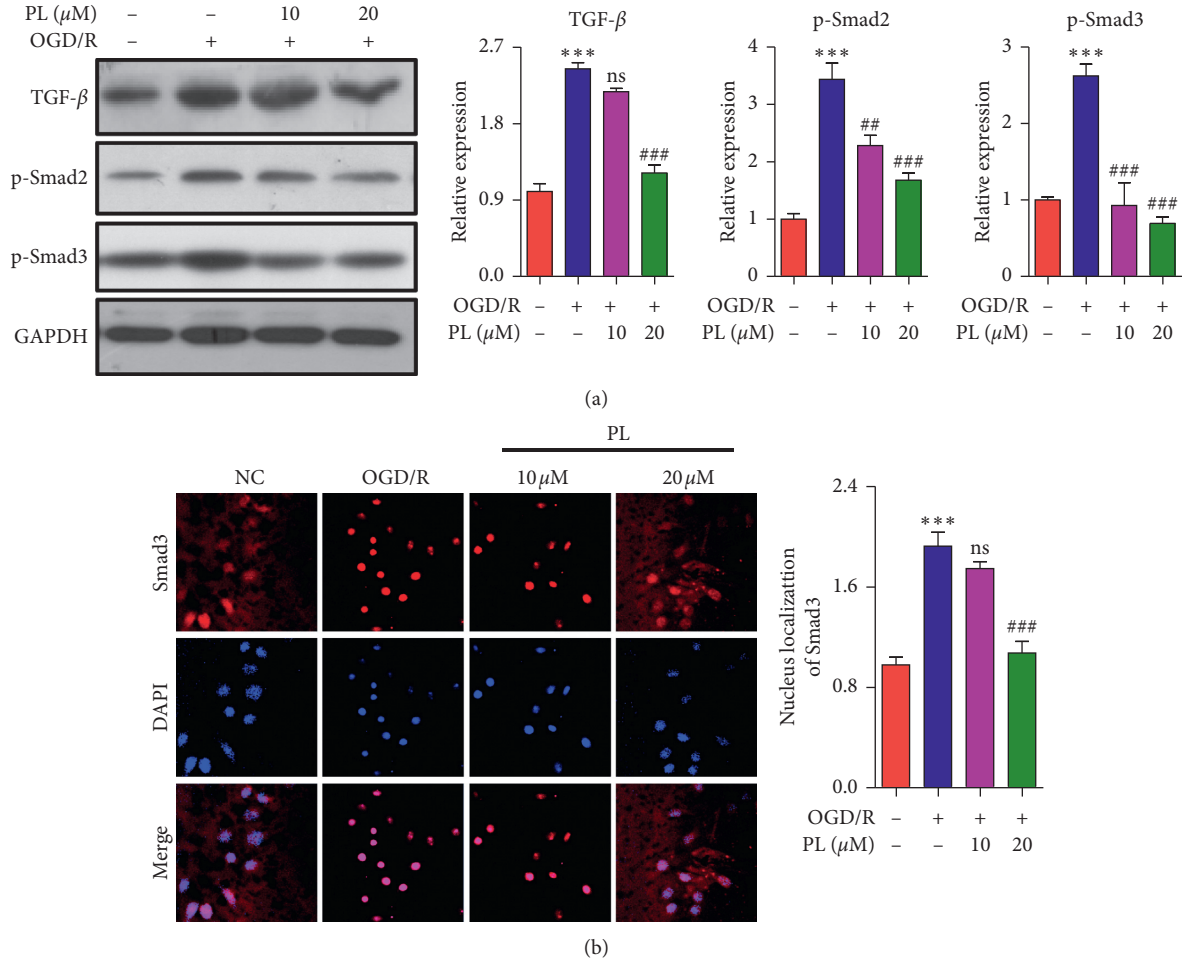

Figure 4: Punicalin mediates the TGF-β/Smad pathway in SH-SY5Y cells. (a) The expression of TGF-β/Smad pathway proteins of TGF-β and p-Smad2/p-Smad3. (b) Immunofluorescence images of Smad3 for nucleus translocation. PL, punicalin. $^{***}p < 0.001$ compared to the normal control, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the OGD/R group, and ns represents not significant.

reperfusion injury and its underlying mechanism is poorly understood. In this research, we found that punicalin might alleviate the OGD/R-induced cellular injury via TGF-β pathway-mediated oxidative stress and cell cycle in SH-SY5Y cells.

The TGF-β/Smad pathway widely exists in the variety of tissues and cells, which plays a crucial role in the progression of cancer, diabetes, and cerebral injury. The TGF-β/Smad pathway induces the phosphorylation of Smad2/smad3 and triggers the shift of Smad3 from the cytoplasm to the nucleus, following evokes related gene expression, and contributes to the pathological process. In the present research, we found OGD/R induced the expression of TGF-β, the phosphorylation of Smad2/Smad3, and nucleus translocation in SH-SY5Y cells; meanwhile, punicalin reversed these changing trends. The results demonstrated that the TGF-β/Smad pathway might take part in the OGD/R-induced cellular injury and punicalin could alleviate the cellular injury via the TGF-β/Smad pathway.

The cell cycle is characterized by a biological process dividing a cell into two daughter cells, which involves the plentiful of regulatory protein and programmed events including the expression and interaction of CDKs and cyclin [24]. It participates in all of the physiology behaviors of differentiation, proliferation, development, and growth from the fertilized eggs and embryo to the individual death [24–26]. The dysregulation of cell cycle would lead to many diseases of cancer and ischemia/reperfusion injury [27–29]. Several shreds of evidence have also shown that the TGF-β/Smad pathway could exert the regulatory effect of cell cycle to control cell proliferation and cell death [30, 31]. In the current research, cell cycle distribution analysis displayed that OGD/R induced the cell cycle G1 phase arrest and punicalin could alleviate the induced cell cycle arrest. It was further confirmed that punicalin could reverse OGD/R-regulated expression of CDK1 and cyclin B1, the cell cycle G1 phase-related protein. Combined with the above data of the TGF-β/Smad pathway, it is speculated that the TGF-β/Smad pathway might involve in the OGD/R-induced cell cycle G1 arrest, and punicalin could alleviate OGD/R-evoked cellular injury via the TGF-β/Smad pathway-mediated cell cycle arrest.

Oxidative stress is considered as one of the critical contributions in individual aging and disease progression. When the balance of oxidization in the body is tipped, it would trigger

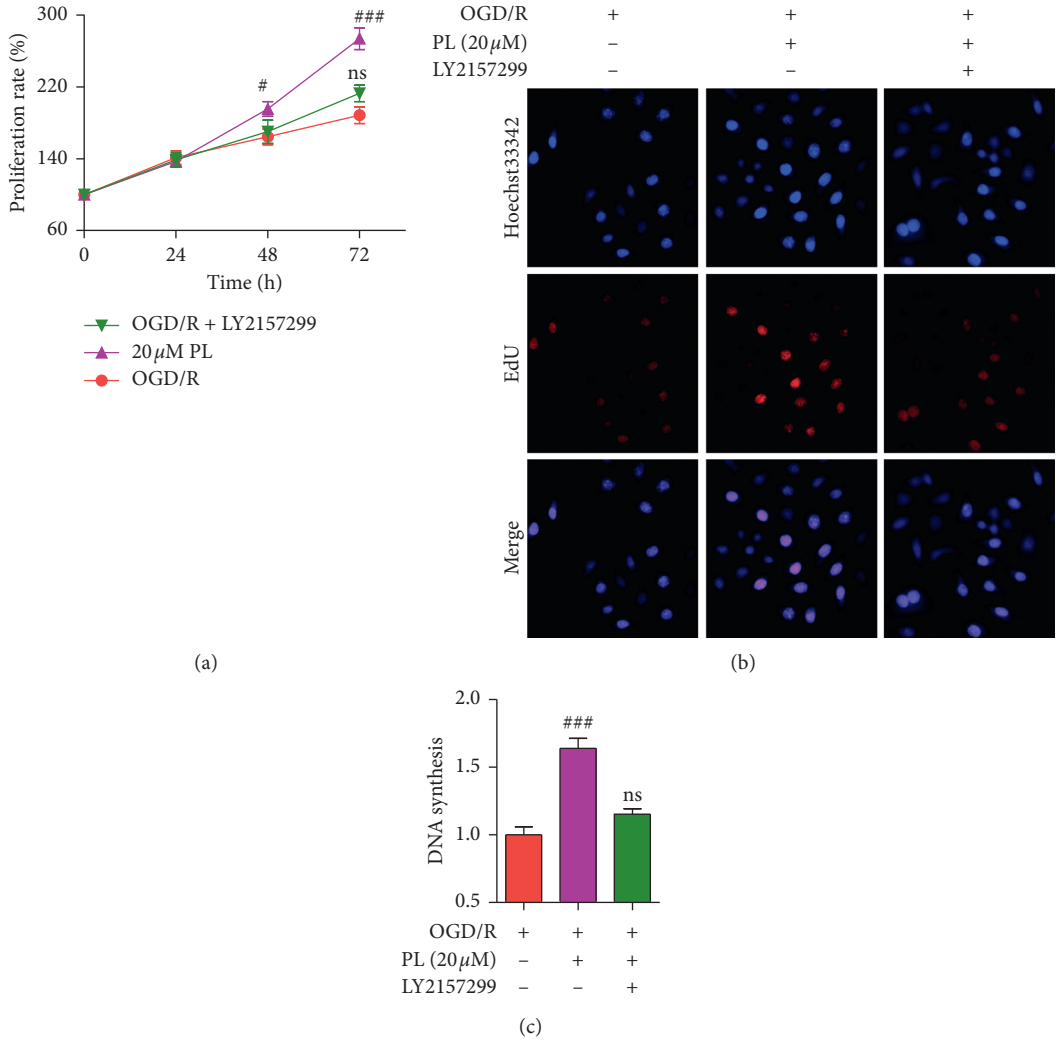

Figure 5: TGF-β/Smad pathway is crucial to the effect of punicalin on OGD/R-induced neuroblastoma injury. (a) The cell proliferation rate of cell subjecting OGD/R, punicalin, and LY2157299. (b) The images of EdU staining for DNA synthesis rate in SH-SY5Y cells (c) The statistics of DNA synthesis rate. LY2157299, an inhibitor of TGF-β/Smad pathway. PL, punicalin. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to the OGD/R group, and ns represents not significant.

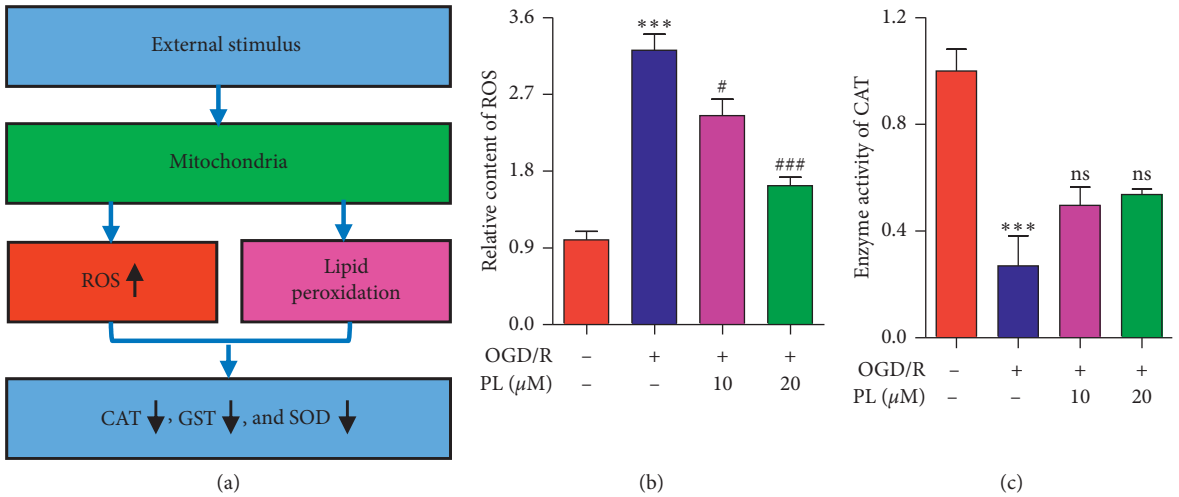

Figure 6: Continued.

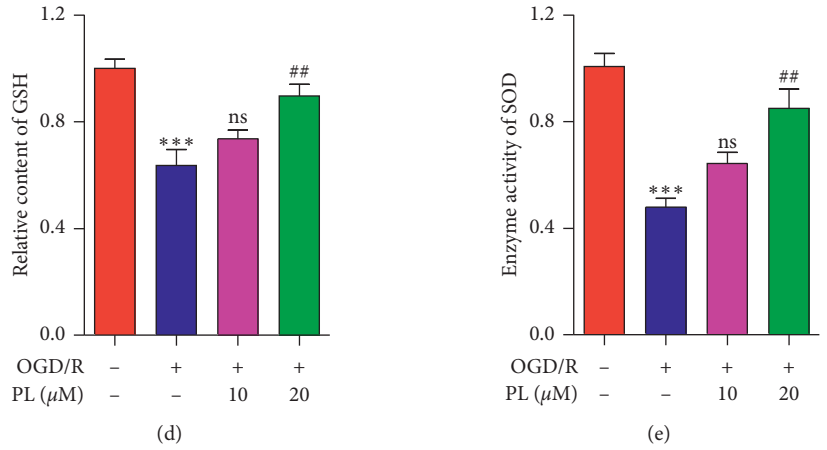

Figure 6: Punicalin alleviates OGD/R-induced oxidative stress in SH-SY5Y cells. (a) The mechanism of oxidative stress. (b) The relative content of ROS. (c) The enzyme activities of CAT. (d) The relative content of GSH. (e) The enzyme activity of SOD. PL, punicalin. $^{***}p < 0.001$ compared to the normal control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared to the OGD/R group, and ns represents not significant.

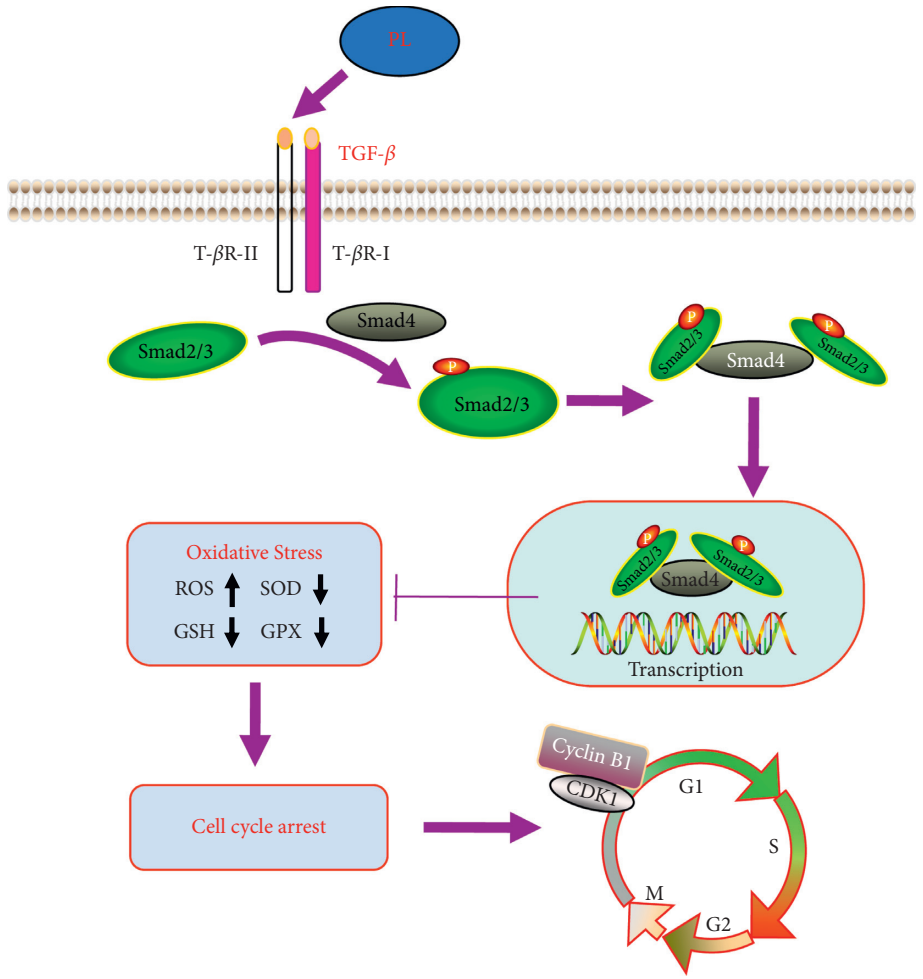

Figure 7: The mechanism of punicalin attenuating OGD/R-induced neuroblastoma injury. PL, punicalin.

numerous of infiltrating inflammation of neutrophil, produce many oxygen free radicals, and cause the dysfunction of macromolecules including protein, DNA, and lipid, named oxidative stress. Increased ROS content, decreased GSH content, and SOD enzyme activity are the indicators of oxidative stress. Emerging shreds of evidence showed that ischemia/reperfusion injury is associated with oxidative stress. Many agents, including dioscin, α-tocopherol, and irisin, were reported to exert protective effects against ischemia/reperfusion injury via oxidative stress [32–34].

Additionally, there is an acceptable crosstalk between oxidative stress and the TGF-β/Smad pathway. It had been reported that the TGF-β/Smad pathway could regulate many pathological behaviors related to oxidative stress injury. Our data displayed that OGD/R raised the ROS content and inhibited the GSH content and the enzyme activities of CAT and SOD, while punicalin relieved the regulation of OGD/R in SH-SY5Y cells. Together, these results revealed that punicalin might alleviate OGD/R-evoked cellular injury via TGF-β/Smad pathway-mediated oxidative stress.

5. Conclusions

In conclusion, the present research identified punicalin as the active component from *Punica granatum* L. to protect against OGD/R-induced cell injury and clarified the underlying mechanism that punicalin could alleviate OGD/R-induced cell injury via TGF-β/Smad pathway-regulatory oxidative stress and cell cycle arrest. The research expands the nutritive value of *Punica granatum* L., improves the understanding to the bioactivities and mechanism of punicalin, and provides a potential and alternative choice to the prevention and treatment to ischemia/reperfusion injury.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Ginsenoside Rb3 Alleviates the Toxic Effect of Cisplatin on the Kidney during Its Treatment to Oral Cancer via TGF-β-Mediated Mitochondrial Apoptosis

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Objective. The research aimed to confirm the role of the transforming growth factor-β (TGF-β) in cisplatin- (CPT-) evoked kidney toxicity and elucidate the mechanism that ginsenoside Rb3 (Rb3) could alleviate the kidney toxicity by CPT during its treatment to oral cancer via TGF-β-mediated mitochondrial apoptosis. *Methods*. The model of xenograft nude mice bearing oral carcinoma cells ACC83 was established and treated with CPT and/or Rb3, respectively. Bodyweights of the treated mice were weighed, and the kidney tissues were collected; following, the histopathology and the expression of TGF-β were examined using H&E staining and immunohistochemistry. Afterward, the renal cells GP-293 were treated with CPT and/or Rb3. The expression and phosphoration of TGF-β, Smad2, Smad3, Bcl-2, and Bax in GP-293 cells were detected by Western blotting. The cellular apoptosis and mitochondrial membrane potential were analyzed using flow cytometry. *Results*. The xenograft nude mice exposure to CPT presented the bodyweight loss, necrotic areas, and the increased expression of TGF in kidney tissue, and Rb3 pretreatment relieved these changes evoked by CPT. In GP-293 cells, CPT administration induced the phosphorylation of Smad2 and Smad3, and Rb3 pretreatment suppressed the induced phosphorylation by CPT. Besides, flow cytometry analysis showed that Rb3 inhibited the CPT-evoked cellular apoptosis ratio and mitochondrial membrane depolarization. The Western blotting test indicated that Rb3 alleviated the cleavage of PARP, caspase 3, caspase 8, and caspase 9, the induction of Bax expression, and inhibition of Bcl-2 expression. Additionally, after treating with the TGF inhibitor of disitertide, Rb3 exhibited no alleviation effects on CPT-evoked cellular apoptosis ratio, inhibition of Bax expression, and induction of Bcl-2 expression in GP-293 cells. *Conclusion*. Rb3 could alleviate CPT-evoked toxic effects on kidney cells during its treatment to oral cancer via TGF-β-mediated mitochondrial apoptosis.

1. Introduction

Oral cancer, occurring in the oral cavity and lip, is the sixth most common carcinoma and accounts for approximately 300,000 new cases around the earth [1]. The lack of early diagnosis strategy to oral cancer delayed the treatment and led to the dismal survival rates and mortality in oral cancer [2–5]. According to the statistics, there are 145,000 patients of oral cancer who died in 2012 [1]. Oral cancer has caused an alarming mental and health burden in human [6, 7]. Although there are plentiful of therapy methods for oral cancer in clinic and theory, the effective strategy for cancer treatment remains a challenge [8, 9]. Currently, chemotherapy is still one of the usual therapies to various cancers including oral cancer [10], and cisplatin (CPT) is an essential chemotherapeutical agent wildly applied to oral cancer for years [11, 12]. However, CPT could kill almost all of the contacted cells of normal and cancer cells in the body, which lead to the toxicity to normal tissues such as the heart, kidney, and liver [13]. The lack of selectivity to cells restricted the clinic application of CPT in treating against oral cancer [14]. Excitingly, recent research studies have shown that several traditional Chinese herb extractions and their components could suppress CPT-trigged liver impairment, kidney injury, and myocardial damage [15, 16]. Among these active herb extractions and components, it was reported that Rb3 could alleviate CPT-evoked kidney injury in the cancer model [17]. However, the potential effect and mechanism of

Rb3 on the kidney toxicity during CTP treating against oral cancer remain obscure. Therefore, it is urgent to elucidate the kidney protective role of Rb3 in the process of CPT-treating oral cancer for Rb3's medicinal development and the clinic treatment of oral cancer.

TGF-β pathway is considered as an essential process of signal cascade response to the transforming growth to mediate cell proliferation, apoptosis, and differentiation [18]. It exerts a variety of pathological and physiological function in the tumor progression, wound healing, immune response, and so on [19, 20]. Recently, several emerging shreds of evidences have shown that TGF-β could serve as a tissue repair mediator to benefit or exacerbate the tissue damage in the brain, liver, and other tissues via mitochondrial apoptosis [21, 22]. Jin et al. reported that TGF-β contributed to LRG1-induced ischemia/reperfusion injury via apoptosis [23]. Another study by Wang et al. also indicated that TGF-β participated in the miRNA-590-5p-regulated chondrocyte apoptosis in response to the mechanical pressure injury [24]. Mitochondrial apoptosis is exceptionally essential to maintain the stabilization of homeostasis in the body, which could clean up the unwanted and abnormal cells according to a serious of programmed signal instruction of apoptosis factors. However, the disorder and despitefully employed apoptosis would result in normal cell death and tissue injury [25]. In the treatment of cancer by CPT, enormous shreds of evidence confirmed that CPT induced the cell apoptosis and tissue injury in the normal kidney, liver, lung, and other tissues and eventually caused the severe toxicity effect [26]. This information implies that CPT-evoked kidney toxicity during the treatment of oral cancer may be associated to the TGF-β-induced mitochondrial apoptosis.

In the present study, we aimed to confirm the role of TGF-β in the CPT-evoked kidney toxicity during the treatment of oral cancer and elucidate the underlying mechanism that Rb3 alleviates kidney toxicity by CPT via TGF-β-mediated mitochondrial apoptosis. First, the xenograft nude mice model bearing oral carcinoma cells ACC83 was constructed. The kidney toxicity by CPT, the protective effect of Rb3, and the TGF-β expression in kidney tissue were evaluated. Second, the human renal epithelial GP-293 cells were employed to determine the effect of Rb3 and CPT on the TGF-β signal pathway and mitochondrial apoptosis. Finally, the role of the TGF-β signal pathway on Rb3 suppressing mitochondrial apoptosis evoked by CPT in GP-293 cells was studied.

2. Materials and Methods

2.1. Chemicals and Reagents. The standards of ginsenoside Rb3 and cisplatin was obtained from Aladdin (China). Hematoxylin and eosin were purchased from Nanjing Jiancheng (China). The primary antibody of TGF-β, Smad2, Smad3, p-Smad2, p-Smad3, Bcl-2, Bax, cleaved PARP, cleaved caspase 3, cleaved caspase 8, cleaved caspase 9, and β-actin were supplied from Abcam (USA). Annexin V-FITC apoptosis kit was purchased from BD (USA). JC-1 dye was obtained from Solarbio (China). Caspase 3, caspase 8, and caspase 9 colorimetric assay kits were purchased from BioVision (USA).

2.2. Establishing the Xenograft Nude Mice Model Bearing Oral Carcinoma Cells ACC83. The SPF male and 6 weeks aged nude mice were purchased from the Xiamen University Laboratory Animal Center (Xiamen, China). All mice were housed according to genotype and sex, five per cage and raised at the atmosphere of 22–26°C on a 12-hour light-dark cycle with continuous access to food and water. After adapting the environment for five days, the mice were subcutaneously implanted with the precultured oral carcinoma cells ACC83 (approximately 5×10^6 cells in 100 μL PBS per mice). One week later, when the implanted ACC83 cells began to display a little tumor swell with different sizes in the subcutaneous of mice, we selected the mice bearing tumor of uniform size as mung beans and randomly divided into four groups ($n = 6$). The selected mice were maintained for another one week and treated with CPT and/or Rb3 to evaluate the toxic effect.

2.3. Animal Experiments Design and Treatment. The nude mice bearing oral tumor were divided into four groups: control, CPT, CPT + low Rb3, and CPT + high Rb3 groups. The control group was administrated with saline once daily for continuous 28 days. The CPT group was intraperitoneally injected with 10 mg/kg cisplatin every other day for 28 days. The CPT + low Rb3 and CPT + high Rb3 groups were administrated with 10 mg/kg and 20 mg/kg ginsenoside Rb3, respectively, once daily, and the same cisplatin injection for the CPT group for 28 days. Bodyweight of mice was measured once weekly. At the end of the treatment on 28th day, all mice were euthanized, and the kidneys were immediately dissected out and fixed with 4% paraformaldehyde for histopathological analysis.

2.4. H&E Staining for Histopathology. After euthanizing, the kidneys from all groups of nude mice were collected. The left kidneys were washed with precooled phosphate buffer saline (PBS) and stored in an ultralow temperature freezer of −80°C for the following Western blotting experiment. The right kidneys were cut into the squares (0.7 cm × 0.7 cm) and fixed with 4% paraformaldehyde at 4°C for more than 24 hours. The fixed squares of kidneys were embedded in paraffin, sliced in 3 μm thickness, and then adhered to slides. The sliced thin kidney samples were dewaxed via continuously placing in the solution of xylene I for 10 min, xylene II for 10 min, anhydrous alcohol I for 5 min, anhydrous alcohol II for 5 min, 95% alcohol for 5 min, 90% alcohol for 5 min, 80% alcohol for 5 min, and 70% alcohol for 5 min, respectively, and then washed with distilled water. The dewaxed slides were further stained with hematoxylin for 5 min and eosin for 3 min. At last, the stained kidney tissues were dehydrated in the solution of 95% alcohol, anhydrous alcohol, and xylene in tune and coverslipped with neutral resins for detecting and imaging using a fluorescence microscope (Leica, Germany).

2.5. Immunohistochemistry for the Expression of TGF-β. The above dewaxed slices were put into the boiled solution of citrate for antigen retrieval and then blocked in 10% goat serum at room temperature for 1 hour. The blocking slices were added 50 μL diluted solution of a primary antibody of TGF-β (1 : 500 dilution) and incubated at 37°C for 1 hour, washed with PBS for three times followed by the secondary antibody for another 1 hour. After washing with PBS for three times, the slices were colored with 50 μL amount of the fresh chromogenic reagent of DAB and stained with hematoxylin for 30 seconds. The running water washed the slices for 20 minutes to cease the staining. Finally, the stained kidney tissues were dehydrated in the solution of 95% alcohol I for 3 seconds, 95% alcohol II for 3 seconds, anhydrous alcohol for 5 minutes, xylene-alcohol (1 : 1) for 5 minutes, xylene I for 2 minutes, and xylene II for 2 minutes in tune and coverslipped with neutral resins for detecting and imaging using a fluorescence microscope (Leica, Germany).

2.6. Cell Culture and Treatments. The oral carcinoma ACC83 cells and normal renal epithelial cells GP-293 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS in a 5% CO_2 atmosphere at 37°C. When the amounts of ACC cells in the logarithmic phase reached to 12 of 10 cm cell culture plates with 90% confluence, they were digested with 0.25% trypsin solution and resuspended into 5 mL EP tubes with PBS according to 200 μL PBS per 10 cm cell culture plate; following, the resuspended cells were subcutaneously injected into nude mice at 100 μL for one mouse to construct the oral tumor mice model. When GP-293 cells in the logarithmic phase were about 85% confluence, they were seeded into 6 wells cultured plate and treated with CPT and/or Rb3 as necessary. And then, the cells were collected and detected by the following experiments.

2.7. Flow Cytometry of Dual-Staining of FITC-Annexin V/PI for the Cellular Apoptosis. The GP-293 cells treated by CTP and/or Rb3 were digested into single cells with 0.25% trypsin and transferred into 1.5 mL EP tubes. The digested cells were washed with precold PBS and centrifuged at 1500 rpm and 4°C for 5 minutes. The precipitate of cells was resuspended with 300 μL 1 × binding buffer; following, 5 μL Annexin V-FITC was added to incubate at room temperature away from light for 15 minutes, and then, 5 μL of PI was added to stain. At last, the stained cells were supplied with another 200 μL 1 × binding buffer and detected by the flow cytometry (Bechman, USA).

2.8. Western Blotting for Detecting the Expression of the Protein. The GP-293 cells treated by CTP and/or Rb3 were harvested and lysed with RIPA buffer. The total protein concentration of cell lysates was detected using BCA kit and then was denatured with loading buffer under the condition of boiling. 30 μg of the total denatured proteins were loaded and subjected to the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. The separated proteins were transferred to PVDF membrane. After blocking with 5% skim milk in Tris buffered saline with Tween 20 (TBST) for 1 hour, the PVDF membranes were incubated with the primary antibodies against TGF-β, Smad2, Smad3, p-Smad2, p-Smad3, Bcl-2, Bax, cleaved PARP, cleaved caspase 3, cleaved caspase 8, and cleaved caspase 9 at 4°C overnight, and the antibody of β-actin was employed as an internal reference. The PVDF membranes were washed with TBST three times and further incubated with the corresponding secondary antibody at room temperature for 2 hours. Finally, the corresponding target on the PVDF membrane was probed using ECL reagent.

2.9. Flow Cytometry of JC-1 Staining for the Mitochondrial Potential. Following the treatment of CPT and/or Rb3 in GP-293 cells, the medium was discarded, and the cells were washed using PBS, and fresh culture medium (1 mL) was added. Following, 1 mL of JC-1 working solution was added into the cells to incubate at 37°C for additional 20 minutes. After incubation, the cells were washed twice with the precold JC-1 staining buffer and digested with 0.25% trypsin. The digested cells were used for flow cytometry analysis.

2.10. Statistical Analysis. All of the experiments data were presented as the mean ± standard deviation (S.D.). The statistical differences among the groups were compared using one-way ANOVA by SPSS of version 19.0 (SPSS, USA). $p < 0.05$ was considered to be statistically significant. The asterisk (*) represented the comparison with the control group, and the pound sign ($^\#$) was for the comparison with the CPT group.

3. Results

3.1. The Protective Effects of Rb3 on CPT-Evoked Kidney Toxicity in Xenograft Nude Mice. Bodyweight and histopathology are standard methods for the evaluation of medicinal toxicity. In the control group, the bodyweight of xenograft nude mice bearing oral carcinoma cells ACC83 had a linear increase from 0 to 28 days (Figure 1(b)). The mice injected with CPT displayed a horizontal linearity in bodyweight which was evident beneath compared with that of the control group, demonstrating that CPT caused the bodyweight loss of mice. Expectedly, ginsenoside Rb3 (the structure shown in Figure 1(a)) administration by gavage at 20 mg/kg dosage in CPT + Rb3 groups alleviated the loss of bodyweight caused by CPT as determined by the bodyweight curve close to that of the control group. Furthermore, histopathology analysis (Figure 1(c)) showed that numerous necrotic areas were found in the kidney tissue of the CPT group rather than that of the control group. Meanwhile, ginsenoside Rb3 pretreatment inhibited the necrosis of kidney tissue caused by CPT. These results indicated that ginsenoside Rb3 could protect against the CPT-evoked kidney toxicity in xenograft nude mice bearing an oral tumor.

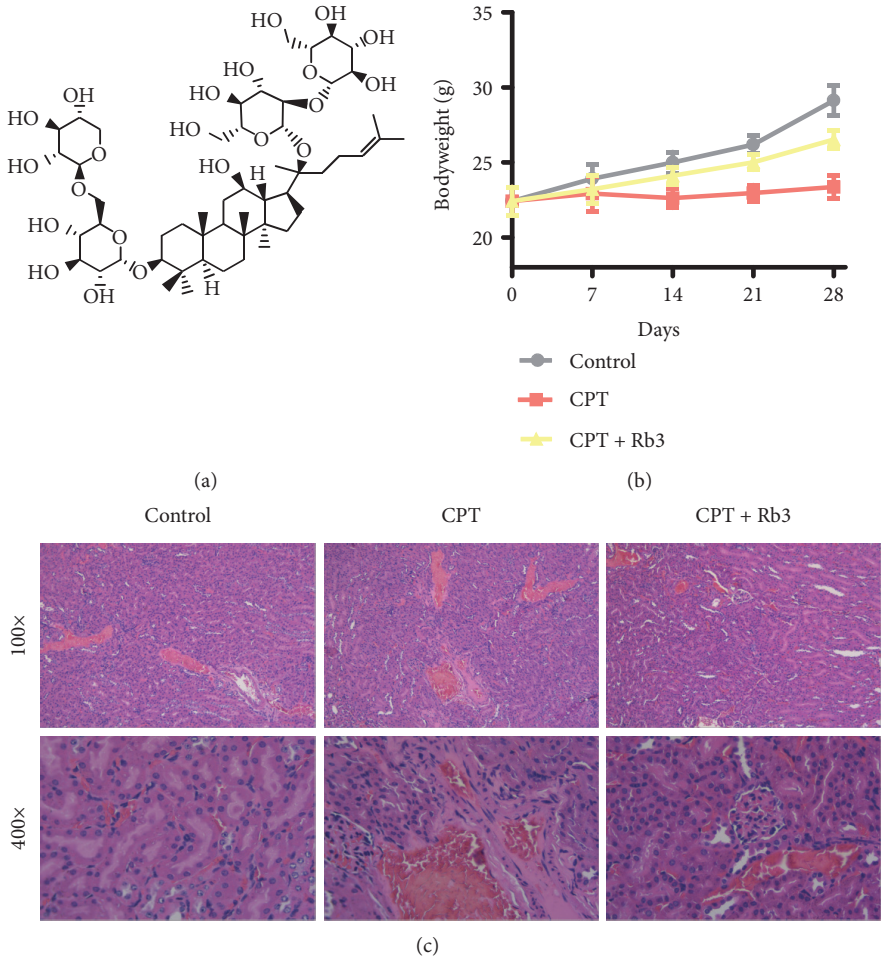


Figure 1: Rb3 protected against CPT-evoked kidney toxicity in xenograft nude mice. (a) The structure of ginsenoside Rb3. (b) The curves of bodyweight of mice. (c) H&E staining of kidney tissue.

3.2. The Effects of Rb3 on the Expression Level of TGF-β in Kidney Tissue of Xenograft Nude Mice. The level of TGF-β expression was examined with immunohistochemical staining and Western blotting. As seen in the representative images of immunohistochemical (Figure 2(a)), kidney tissue of mice in the control group presented a normal cellular morphology and TGF-β expression. In contrast, CPT injection promoted the induction of deformed cellular morphology and TGF-β expression compared with these of the control group ($^{*}p < 0.01$, Figure 2(b)). Conversely, Rb3 administration at 20 mg/kg dose suppressed CPT-induced TGF-β expression and repaired the deformed cellular morphology in kidney tissue, and its levels were similar with these in the control group (Figures 2(a) and 2(b)). Additionally, Western blotting assay revealed the similar changing trend of TGF-β expression with data obtained by immunohistochemical staining, which was that CPT induced the TGF-β expression and Rb3 suppressed the induction of TGF-β expression by CTP in kidney tissues of xenograft nude mice bearing oral tumor.

3.3. The Effects of Rb3 on the TGF-β Signal Pathway in GP-293 Cells. To further evaluate the effect of Rb3 on the TGF-β signal pathway, we detected the expression and phosphorylation level of Smad2 and Smad3 using Western blotting assay in human renal epithelial GP-293 cells. As shown in Figure 3, the expression level of TGF-β of cells exposed to CTP was induced compared with that in the control group which is consistent with the data mice ($^{***}p < 0.001$, Figure 3(b)), meanwhile CTP activated the phosphorylation level of Smad2 ($^{***}p < 0.001$, Figure 3(c)) and Smad3 ($^{***}p < 0.001$, Figure 3(e)) and but no statistical changing in the expression of Smad2 and Smad3. Following the treatment with Rb3 at different concentrations, the TGF-β expression level and phosphorylation level of Smad2 and Smad3 in GP-293 cells were suppressed compared to the CPT group, and its suppression level was concentration-dependent. Together, the data in vivo/in vitro imply that CPT could mediate TGF-β expression and its pathways and Rb3 possesses the suppression abilities to the CPT-mediated TGF-β signal pathway.

3.4. The Effects of Rb3 on CPT-Evoked Cellular Apoptosis in GP-293 Cells. To confirm the role of cellular apoptosis in Rb3-protecting CPT-evoked toxicity, flow cytometry of dual-staining of FITC-Annexin V/PI and Western blotting were performed to detect cellular apoptosis in human renal

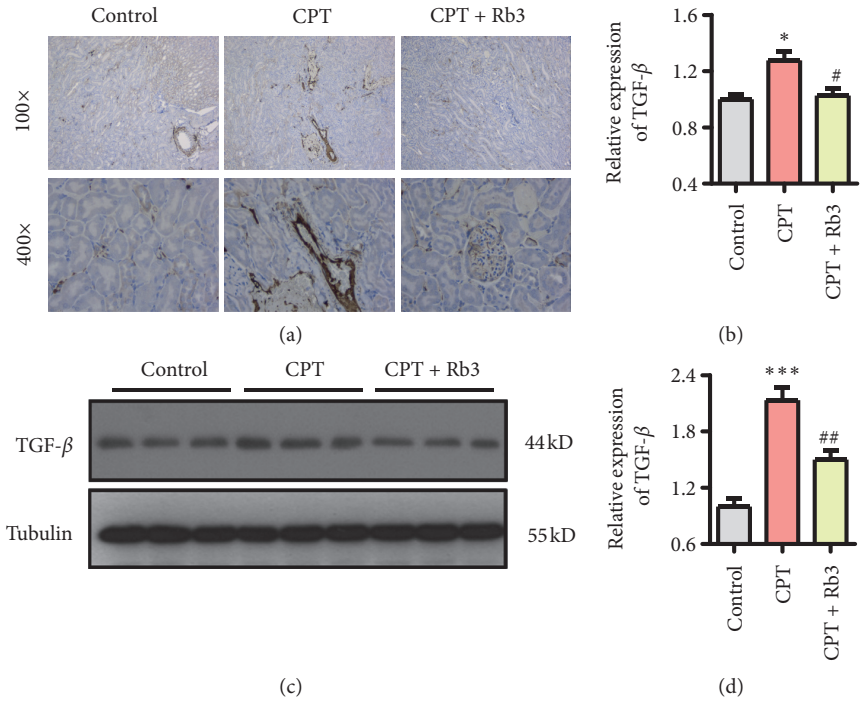
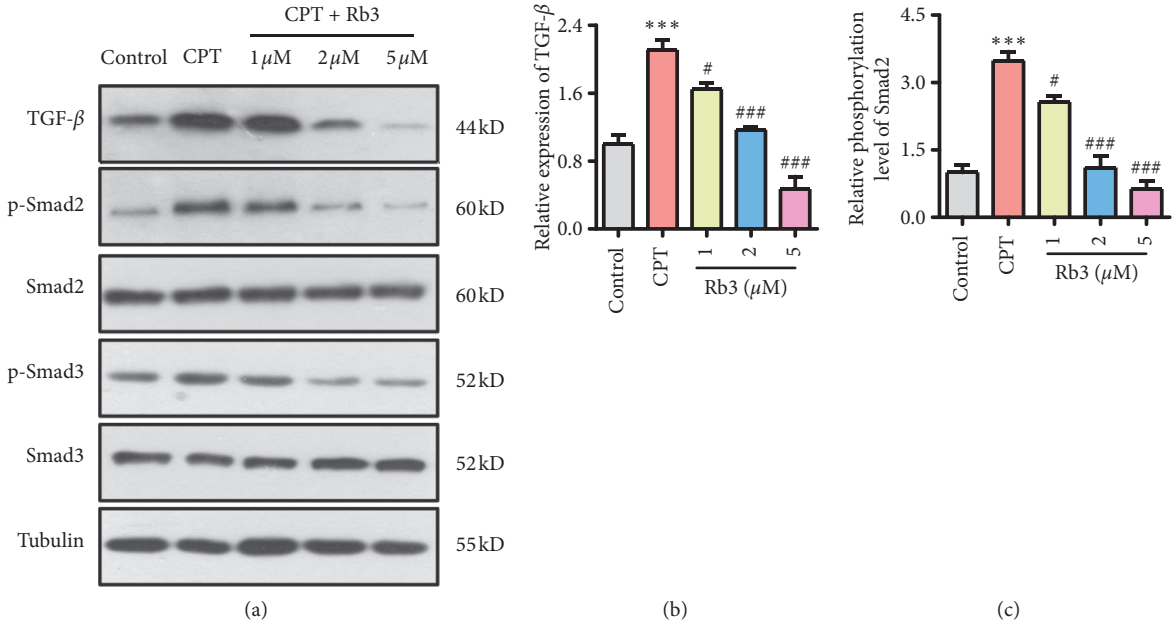


Figure 2: Rb3 suppressed the TGF-β expression evoked by CPT in kidney tissue of xenograft nude mice. (a) Immunohistochemistry for TGF-β expression in kidney tissue. (b) Quantitative analysis of TGF-β expression by immunohistochemistry. (c) Western blotting for TGF-β expression in kidney tissue. (d) Quantitative analysis of TGF-β expression by Western blotting. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the control group; $^{\#}p < 0.05$ compared to the CPT group.



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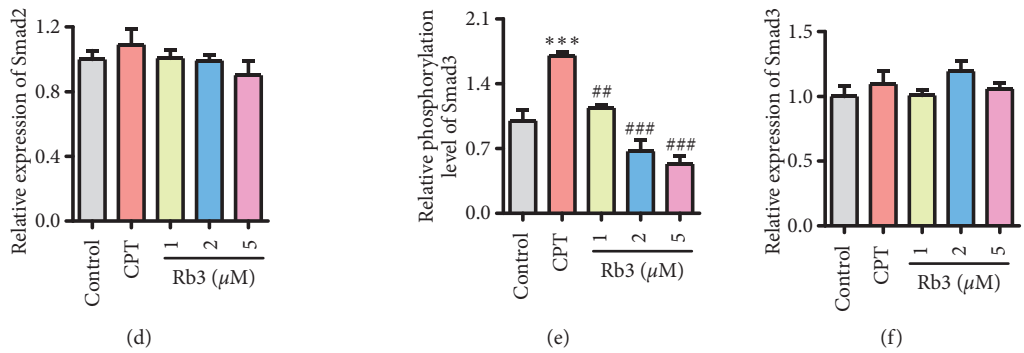


Figure 3: Rb3 regulated the TGF-β signal pathway in GP-293 cells. (a) Western blotting for the expression of TGF-β, Smad2, and Smad3 and phosphorylation of Smad2 and Smad3. (b–e) Quantitative analysis of densitometry for TGF-β (b), p-Smad2 (c), Smad2 (d), p-Smad3 (e), and Smad3 (f). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared to the CPT group.

epithelial GP-293 cells. As shown in the quadrant scatter spot graphs of flow cytometry (Figure 4(a)), the cells in the control group exhibited an apoptosis ratio of only $2.88\% \pm 1.43\%$, while the apoptosis ratio of the cells exposed to CPT rose to $18.10\% \pm 1.28\%$ (Figure 4(b)) and demonstrated that CPT significantly induced the cellular apoptosis in GP-293 cells. After the supplement of Rb3 at different concentrations, the apoptosis ratio of cells gradually decreased with the rising of Rb3 concentration, and the higher dose of $2\,\mu$M and $5\,\mu$M Rb3 had statistical differences in apoptosis ratio compared with that of the CPT group ($^{\#}p < 0.05$ in $2\,\mu$M Rb3 and $^{\#\#\#}p < 0.001$ in $5\,\mu$M Rb3, Figures 4(a) and 4(b)). Furthermore, the cleaved level of PARP (poly ADP-ribose polymerase), a usual and classic indicator of apoptosis, was detected using Western blotting and displayed a consistent with the results of flow cytometry. The cleaved level of PARP in the CPT group was induced compared with that in the control group ($^{***}p < 0.001$), whereas Rb3 reversed the induction of the cleaved PARP level by CPT, and the higher dose is statistically significant differences ($^{\#\#\#}p < 0.001$ in $2\,\mu$M and $5\,\mu$m Rb3 compared to the CPT group, Figures 4(c) and 4(d)). All of these apoptosis results indicated that $2\,\mu$M Rb3 just had initiated the suppression of the apoptosis induced by CPT in human renal epithelial GP-293 cells. Therefore, we choose the concentration of $2\,\mu$M Rb3 for the follow-up experiments.

3.5. The Effects of Rb3 on CPT-Evoked Mitochondrial Apoptosis in GP-293 Cells. In order to determine whether mitochondrial participated in Rb3 suppressing cellular apoptosis evoked by CPT, the mitochondrial membrane potential of GP-293 cells was detected by flow cytometry of JC-1 staining, and mitochondrial-related protein expression of Bax and Bcl-2 was probed by Western blotting. The histogram from flow cytometry analysis (Figures 5(a) and 5(b)) showed that the fluorescence ratio of green/red in the cells of the CPT group is nearly four-folds to that in the control group and demonstrated that CPT induced mitochondrial depolarization which is the early event of cellular apoptosis. Reservedly, the administration of Rb3 at $2\,\mu$M significantly repaired the mitochondrial depolarization induced by CPT, and its fluorescence ratio of green/red is similar to that in the control group. Additionally, Western blot showed that CPT induced the expression of Bax and inhibited Bcl-2 expression; meanwhile, Rb3 relieved the CPT-mediated protein expression of Bax and Bcl-2 in GP-293 cells (Figures 5(c) and 5(d)). Together, these results demonstrated that Rb3 might suppress the CPT-induced apoptosis via mediating mitochondrial depolarization and protein expression of Bcl-2 and Bax in GP-293 cells.

3.6. The Effects of Rb3 on Caspase 3, Caspase 8, and Caspase 9 in GP-293 Cells. Caspases play an essential role in initiating and executing the cellular apoptosis process. The ELISA assay and Western blotting were employed to examine the activities and cleaved level of caspase 3, caspase 8, and caspase 9 in GP-293 cells, and the results are shown in Figure 6. Figure 6(a) displayed that the cells exposed to CPT had an apparent increase of activities in caspase 3 (7.4 folds, $^{***}p < 0.001$), caspase 8 (4.2 folds, $^{***}p < 0.001$), and caspase 9 (3.1 folds, $^{**}p < 0.01$) compared with those of cells in the control group, whereas Rb3 inhibited the activities of the three caspases induced by CPT in the varying degree. Besides, Western blotting showed that CPT induced the cleaved levels of caspases and Rb3 suppressed the induction (Figures 6(b) and 6(c)). The changing trends of the cleaved level of caspase 3, caspase 8, and caspase 9 are consistent with that of caspases activities in Figure 6(a).

3.7. The Roles of the TGF-β Signal Pathway on Rb3 Suppressing Endogenous Apoptosis Evoked by CPT in GP-293 Cells. The information mentioned above has shown that Rb3 could mediate the TGF-β signal pathway and endogenous apoptosis. To illuminate whether the TGF-β signal pathway is essential to Rb3 suppressing endogenous apoptosis evoked by CPT, we further used the TGF-β pathway inhibitor of disitertide in human renal epithelial GP-293 cells. As shown in Figure 7(a), the apoptosis ratio of the cells under the condition of CPT treatment was $24.3 \pm 4.1\%$ and Rb3 administration significantly suppressed the cellular apoptosis

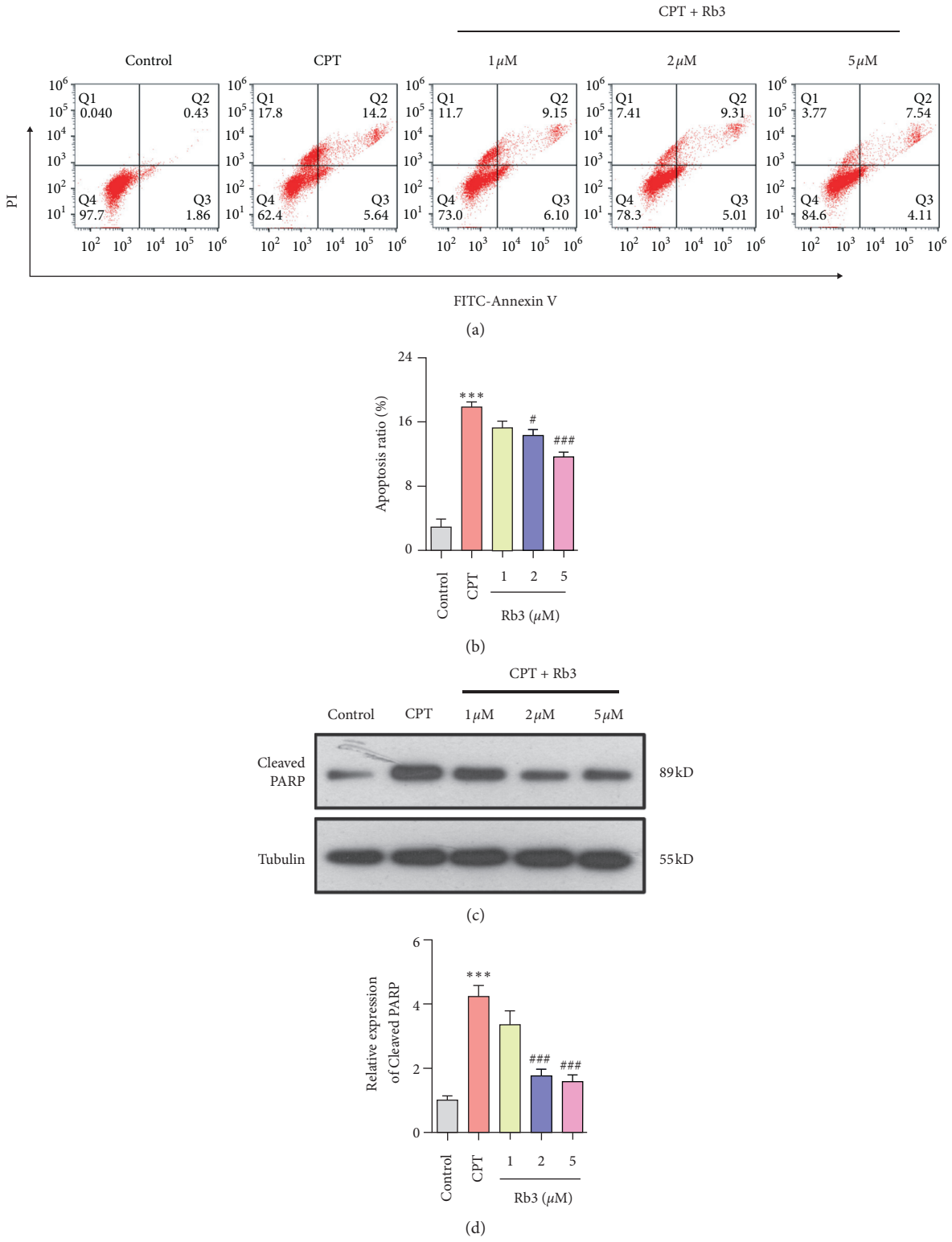


Figure 4: Rb3 suppressed CPT-evoked cellular apoptosis in GP-293 cells. (a) Flow cytometry of dual-stains of FITC-Annexin V and PI for cell apoptosis ratio. (b) Quantitative analysis for the apoptosis ratio by flow cytometry. (c) Western blotting for the cleavage level of PARP. (d) Quantitative analysis of densitometry for the cleaved PARP. $^{***}p < 0.001$ compared to the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the CPT group.

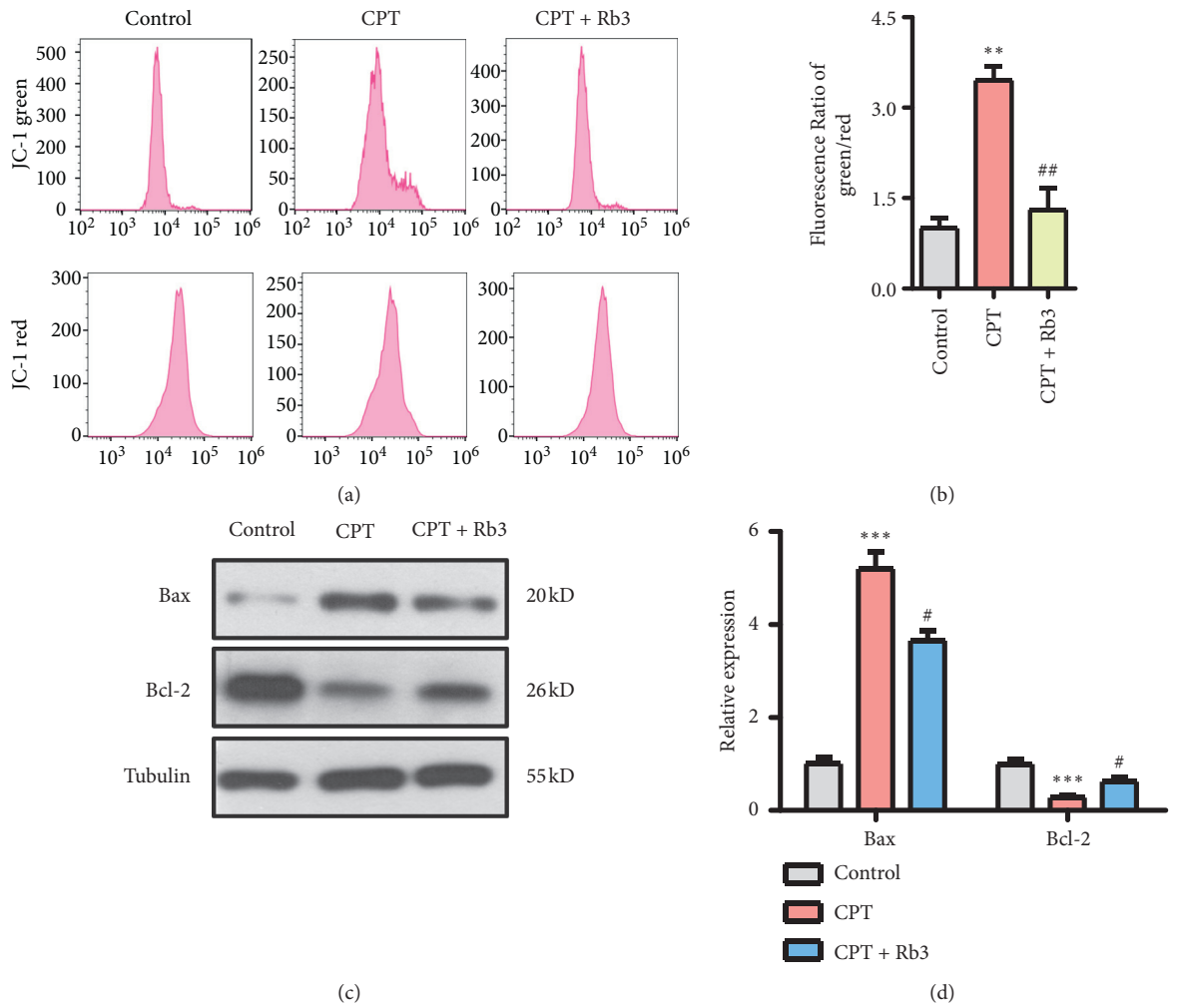
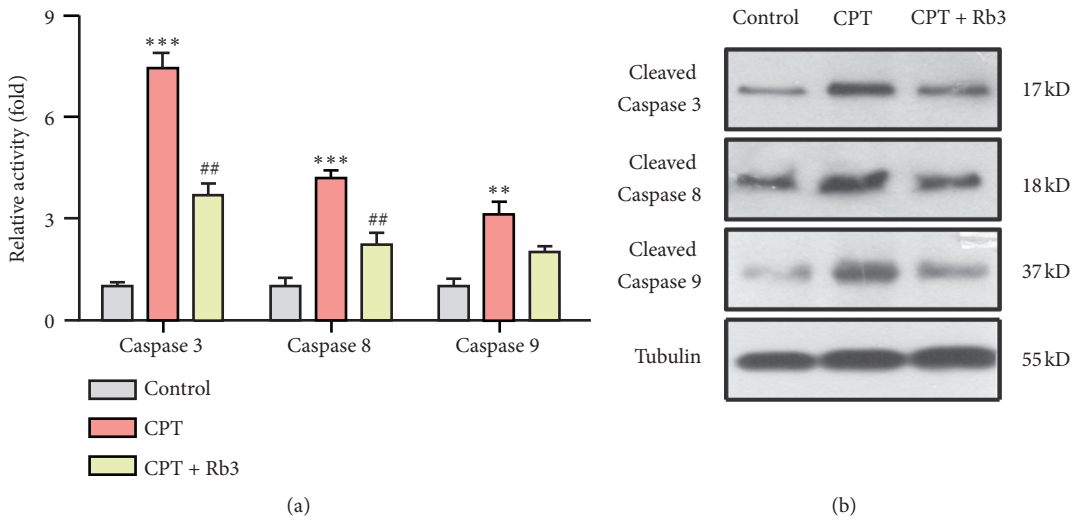


Figure 5: Rb3 suppressed CPT-evoked mitochondrial apoptosis in GP-293 cells. (a) Flow cytometry of JC-1 staining for mitochondrial membrane potential change. (b) Quantitative analysis for mitochondrial membrane potential change. (c) Western blotting for the expression levels of Bax and Bcl-2. (d) Quantitative analysis of densitometry for the expression of Bax and Bcl-2. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the CPT group.



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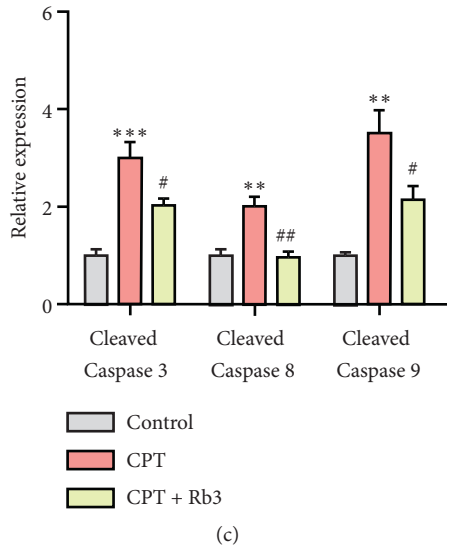

Figure 6: Rb3 regulated caspase 3, caspase 8, and caspase 9 in GP-293 cells. (a) Elisa assay for the activities of caspase 3, caspase 8, and caspase 9. (b) Western blotting for the cleavage levels of caspase 3, caspase 8, and caspase 9. (c) Quantitative analysis of densitometry for the cleavage levels of caspase 3, caspase 8, and caspase 9. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the CPT group.

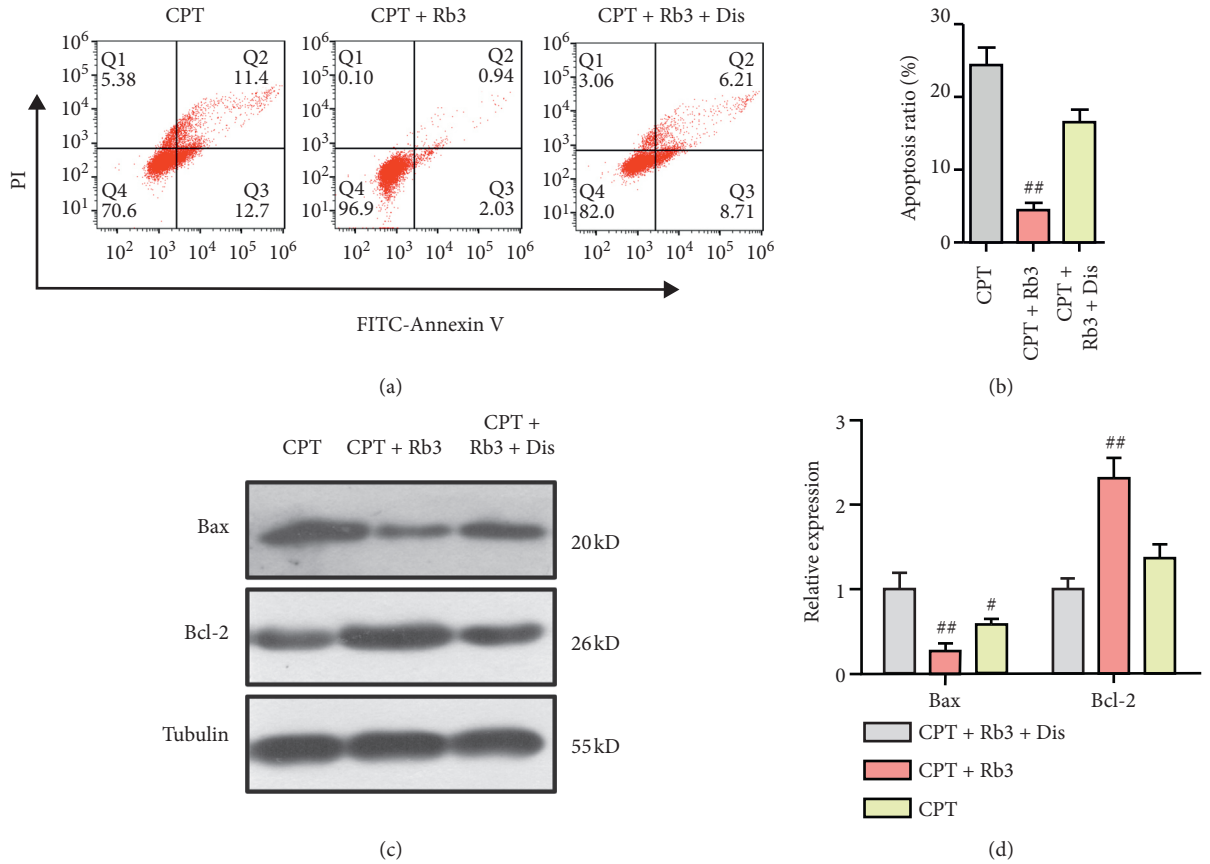

Figure 7: TGF-β signal pathway was essential to that Rb3 suppressed mitochondrial apoptosis evoked by CPT in GP-293 cells. (a) Flow cytometry of dual-stains of FITC-Annexin V and PI for cell apoptosis ratio. (b) Quantitative analysis for the apoptosis ratio by flow cytometry. (c) Western blotting for the expression level of Bax and Bcl-2. (d) Quantitative analysis of densitometry for the expression of Bax and Bcl-2. Dis, disitertide, an inhibitor of the TGF-β signal pathway. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the CPT group.

ratio to 4.3 ± 1.9%. However, after pretreatment with disitertide and Rb3, the cellular apoptosis ratio of 18.0 ± 3.1% was not a statistical decrease compared with that in the CPT group, which was opposite to that only Rb3 administration could just relieve the apoptosis ratio evoked by CPT. Moreover, Western blotting assay showed that Rb3 inhibited the expression of Bax and induced Bcl-2 expression compared with the CPT group, while combinational administration of Rb3 and disitertide deprived Rb3 of the rights mediating Bax and Bcl-2 expression. These results demonstrated that the TGF-β signal pathway is essential to Rb3 suppressing endogenous apoptosis evoked by CPT.

4. Discussion

CPT has been commonly used in the treatment of oral cancer clinically for several decades; however, the toxicity to normal tissue including the kidney limited its benefits as an effective antitumor agent. Although it was in the vague understanding that how CPT induced kidney toxicity, some shreds of evidence have shown that the pathway of NF-κB, autophagy, oxidative stress, and apoptosis are associated with the kidney toxicity by CPT. Additionally, the TGF-β pathway plays a vital role in the kidney injury via kidney fibrosis, macrophage infiltration, and cellular apoptosis. However, the role of TGF-β in CPT-evoked kidney toxicity is rarely reported. In the present study, we found that CPT could induce the expression of TGF-β in the kidney tissue of the xenograft nude mice model bearing oral tumor and in the human renal epithelial cells GP-293; furthermore, CPT could also mediate the TGF-β signal pathway characterizing by the expression and phosphorylation of Smad2 and Smad3 in GP-293 cells. The results demonstrated that TGF-β might take part in CPT-evoked kidney toxicity.

Rb3 is a representative triterpenoid saponin isolated from ginseng, which could exert various biological activities in the apoptosis, oxidative stress, inflammation, and others. Recent studies suggested that Rb3 could serve as a protective agent to alleviate the injury of kidney tissue, cardiomyocyte, and hippocampus [27, 28]. The study by Xing et al. revealed that Rb3 protected against the CPT-induced kidney toxicity via the autophagy pathway mediated by AMPK and apoptosis [17]. In another study, Rb3 was reported to exert the cardioprotective effects via the activation of the antioxidation signaling pathway of PERK/Nrf2/HMOX1 in vivo and vitro [29]. In the current research, we illustrated a novel mechanism that Rb3 might protect against CPT-evoked kidney toxicity via the TGF-β signal pathway in vivo and in vitro. The mechanism confirmed that Rb3 suppressed the expression of TGF-β induced by CPT in the kidney tissue of the xenograft nude mice model bearing oral tumor and in renal cells GP-293 and phosphorylation of Smad2 and Smad3 in GP-293 cells.

Apoptosis is a physiologically programmed cell death process to get rid of the unwanted and dysfunctional cells for maintaining the homeostasis of the body [30]. Mitochondrial, recognized as the regulated center of cellular activities, controls the respiratory chain and oxidative phosphorylation serving for nearly all of the cellular physiology. In the apoptosis progression, mitochondrial depolarization occurs as response to apoptosis stimuli, cytochrome C releases from mitochondrial, and the expression of apoptosis-related factor Bcl-2 and Bax in mitochondrial changes; following actives, the series of cysteinyl aspartate specific proteinase (caspase) of caspase 3, caspase 8, and caspase 9 eventually induced the cleavage of poly ADP-ribose polymerase (PARP) and cell death [31]. Any disordered mitochondrial apoptosis section would cause functional cell death and trigger the normal tissue injury [32]. The research studies had confirmed that CPT evoked the kidney cell apoptosis via inducing Bax expression, inhibiting Bcl-2 expression, and activating caspase activities. TGF-β was also reported to take part in the cell apoptosis via mitochondrial. Our results implied that TGF-β partly contributed to the CPT-induced mitochondrial apoptosis. Moreover, the present study revealed that TGF-β is essential to Rb3 protecting against CPT-evoked apoptosis, which confirmed that Rb3 lost the protective effect in the pretreatment with the TGF-β inhibitor of disitertide in kidney cells.

5. Conclusions

In conclusion, the TGF-β pathway might contribute to CPT-evoked kidney toxicity, and Rb3 could exert a protective effect on the kidney toxicity through the TGF-β pathway-mediated mitochondrial apoptosis during the treatment of oral cancer by CPT. Our finding provides a novel insight into the protective effect of Rb3 on CPT-evoked kidney toxicity. It is very significant for the development of Rb3 and the treatment of oral cancer in the clinic.

Data Availability

The data generated or analyzed to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Review Article

Pharmacological Activity of Eriodictyol: The Major Natural Polyphenolic Flavanone

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Eriodictyol is a flavonoid that belongs to a subclass of flavanones and is widespread in citrus fruits, vegetables, and medicinally important plants. Eriodictyol has been anticipated to explain the method of its activity via multiple cellular signaling cascades. Eriodictyol is an effective natural drug source to maintain higher health standards due to its excellent therapeutic roles in neuroprotection, cardioprotective activity, hepatoprotective activity, antidiabetes and obesity, and skin protection and having highly analgesic, antioxidant, and anti-inflammatory effects, antipyretic and antinociceptive actions, antitumor activity, and much more. This review aims to highlight the modes of action of eriodictyol against various diseases via multiple cellular signaling pathways.

1. Introduction

The flavonoid eriodictyol (ER) [(S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one] (Figure 1) belongs to flavanones subclass [1] mostly found in citrus fruits, vegetables, and most of the medicinal plants [2]. Flavonoids are the polyphenols essentially present in human diet on regular basis and mainly occur in natural herbs [3]. Phytomedicine is an essential part of the traditional Chinese medication system which basically works by scavenging free radical and possesses anti-inflammatory effects. It is assumed that phytochemicals have a noteworthy part in human healthcare systems and account for over 40% of professionally prescribed medicines that are essentially homegrown [4]. Therapeutically, phytomedicine has plenty of advantages over synthetic drugs in terms of low toxicity with reduced or none of the side effects [5]. Phytomedicine is an important source for the exploration of new drugs that help cure various health issues and diseases [6].

ER potentially drives a huge number of cellular signaling pathways to cure different diseases due to its numerous therapeutic effects reported for different ailments. ER is a vital part of dietary supplements and being a part of food poses extraordinary antioxidant effects for reducing the risks of any health issues [7].

2. Sources of Eriodictyol (ER)

ER is a flavanone mainly extracted from yerba Santa Clause (*Eriodictyon californicum*), a plant local to North America [8]. ER is one of the four flavanones isolated from this plant as having taste-altering properties, the other three being

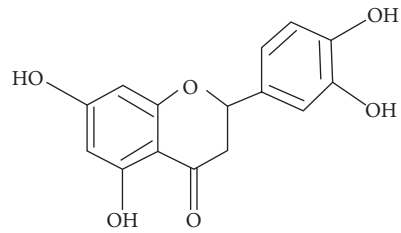

Figure 1: The chemical structure of eriodictyol.

sterubin, homoeriodictyol, and its sodium salt [9]. ER is likewise found in *Eupatorium arnottianum* [10], its glycosides (eriocitrin) in lemons and *Rosa canina* [11], and in the twigs of *Millettia duchesnei* [9]. ER has also been reported to be extracted from the stem bark of *Piptadeniastrum africanum* plant, which is widely used in African traditional remedies [12].

3. Pharmacological Properties

A vast majority of flavonoids present in plants are complexed with carbohydrates, e.g., β-glycosides. The flavonoid ER generally retained as such that gradually expended in the digestive tract as glucuronidation following the action of gut microbiota and is metabolized through methoxylation in the liver and turned into restructured homoeriodictyol. Formed metabolites are in the long run used in the kidneys, delivering glucuronic acids having corrosive or potential sulfate groups from them and enhancing their biliary and urinary discharge. ER as a formed structure has also been identified in blood plasma and urine samples at 4 h and 24 h, respectively [13]. Moreover, ER has a broad spectrum of pharmacological activities, and it was recently reported that its roles in curing various diseases has drawn much attention for this compound.

3.1. Protection in Cardiovascular Issues. Coronary illness is created because of excessive accumulation of oxidative stress generated by various reactive oxygen species (ROS), and low-density lipoprotein (LDL) cholesterol. Nonetheless, it is also demonstrated that ER reduces the chances of myocardial ischemia/reperfusion (I/R) injury in the SD rodent by stifling support of incendiary and myocardial apoptosis responses by modulating Janus kinase 2 (JAK2) pathway [14]. In continuation to this, ER treatment helps improve the cardiomyocyte injury mainly by the activation of the B cell lymphoma-2 (Bcl-2) and Bcl-2-related factor X (BAX) signaling pathway via caspase-3 signaling pathway [15]. Moreover, ER diminishes the activation of C-responsive protein, p38, and JNK2 articulation, which leads to a decrease in NO accumulation and a decrease in expression of VEGF and endothelial bond factor articulation eventually reduces the scar formation and vascular stenosis gives rise to atherosclerosis [16]. In addition, ER triggers ERK/Nrf2/ARE intervened upregulation of heme oxygenase (HO-1) expression in human endothelial cells which is legitimately linked with vascular stability against oxidative stress mediated endothelial injury. This recommends that ER-mediated regulation of HO-1 expression is a promising tool for combating cardiovascular ailments [13].

3.2. Skin Protection. Skin is the largest organ of the human body which is constantly exposed to external environment. Atopic dermatitis is chronic but inflammatory dermatological condition having some allergic signs as well. ER restrains immunoglobulin E (IgE)/Ag-induced type I hypersensitivity mainly via reducing the expression of certain inflammatory interleukin-4 (IL-4) along with ceramide kinase [17]. The extracts of *Aspalathus linearis* (Rooibos) triggers muscarinic M3 receptor to increase the dryness in the skins, as dryness may influence life style and pleasure.

Moreover, eriodictyol-6-C-β-D-glucoside (E6CG) dynamically influences the secretions from exocrine organs. The pharmacokinetics of the dissemination of E6CG in exocrine organs have not been fully elucidated in the mice exposed to rooibos extracts. LC–MS/MS was implied to distinguish E6CG without any change in the structural change and applied on C57BL/6 mice that showed rooibos extracts helping in moving E6CG into the blood plasma shortly after the exposure. Huge amounts of E6CG are present in the submandibular, sublingual, parotid, and lacrimal organs and in the perspiration organs in palm skin. This reports the mechanism of rooibos mediated separation of E6CG in the skin may be utilized in medicated personal care products for the nourishment and improving dryness of the skin [18].

3.3. Dryness of Mouth, Eye, and Skin. Various foods containing rooibos extracts rich in E6CG have significantly reduced dryness of human oral cavity ($P = 0.019$), eyes ($P = 0.006$), and skin ($P = 0.030$) after about fourteen days of application at a 100 mg concentration (0.21 mg E6CG) of crude rooibos dosage. The clinical examinations showed that continuous consumption of rooibos extracts significantly removed dryness of mouth, eye, and skin [19].

3.4. Antitumor Activity. The uncontrolled growth of the cells leads to the formation of tumors. Flavonoids are involved in various cellular pathways for lowering malignant growths of tumors. The anticancer potential of flavonoids may incur at multiple steps via the hindrance in cell development, multiplication, and metastases even [20]. A recent study demonstrated that ER applies its anticancer effects by inducing apoptosis, cell cycle arrest at G2/M phase through restraining the mTOR/PI3K/Akt cascade [21], showing the

involvement of ER through some planned effect especially in the treatment of lung carcinoma. Additionally, ER represses lipid peroxidation and a modulatory effect on preneoplastic injuries in colon by its cytoprotective potential [22].

The downregulation of PI3K/Akt signaling has a basic role in cell proliferation, survival, and autophagy [23]. The increased downregulation in PI3K/Akt expression leads to reduced tumorigenesis [24]. The upregulation in the expression of PI3K pathway may lead to enhanced malignancy by increasing cell proliferation and make the tumor cell resistant by avoiding apoptosis [25]. The NF-κB signaling pathway is of extreme importance that directs different cellular cascades activation to induce disease advancement and tumorigenesis [26]. Upregulation of the NF-κB expression promptly leads to its movement from cytoplasm into the nucleus which may advance malignant growth metastasis [27]. In this regard, focusing on modulation of the PI3K/Akt/NF-κB signaling pathway may be a promising approach while treating the malignant growth. ER could efficiently affect insulin-mediated glucose take-up in human hepatocellular carcinoma cells by controlling the PI3K/Akt signaling pathway [28].

Moreover, ER was found to diminish nitric oxide (NO), TNF-α, IL-6, and IL-1β creation in LPS-activated RAW264.7 cells through downregulation of the MAPK/NF-κB signaling pathways [29]. Herein, the manuscript illustrates that the reducing effects of ER on the glioma may be the suppression of the PI3K/Akt/NF-κB pathway. The study shows that ER may partly induce apoptosis by repressing the phosphorylation of PI3K, Akt, and NF-κB. Moreover, ER inactivates PI3K/Akt/NF-κB pathway, upregulates Bax, and cleaves PARP expression. The involvement of ER in the pro-apoptotic pathways suggests that the former actively triggers apoptosis in U87MG and CHG-5 cells mainly by suppressing the PI3K/Akt/NF-κB pathway [30].

3.5. Effect on Central Nervous System (CNS). Stroke is an unpredictable problem that happens due to excessive oxidative pressure associated pathways in its pathogenesis. The nuclear factor erythroid-2-related factor 2/antioxidant response elements (Nrf2/ARE) pathway is an important signaling mechanism involved in activating phase-II detoxification enzymes along with cytoprotective proteins taking part in neuroprotection during stroke. Being a novel activator of Nrf2, eriodictyol-7-O-glucoside (E7G) ensures protection for cerebral ischemic injury through its Nrf2/ARE pathway in neuroprotection. In cultured astrocytes, E7G enhances the nuclear translocation of Nrf2 and triggers the activation of downstream Nrf2/ARE genes and provides antioxidant responses. These neuroprotection effects are shown by the activation of Nrf2/ARE antioxidant pathway triggered by E7G which is linked with direct reduction of oxidative pressure prompted ischemic injury and might be a promising way out for successful intercession in stroke [31].

3.6. Antioxidant Activity. The potential of scavenging the free ROS or RNA is termed as the antioxidant potential of any compound. The extracts rich in flavonoid can chelate metal particles and free radicals and associate with the metabolic products to provide a vital protection against various cellular degradation processes [32]. A recent study unleashed how ER diminishes lipid peroxidation in isoproterenol-induced myocardial infarcted rodents after 45 days of exposure to the drug [33]. ER provides increased cell protection by enhanced antioxidant activity. In an *in vivo* study, ER showed a decreased oxidative damage in human retinal pigment epithelial- (ARPE-) 19 cells by Nrf2 and the activation of its downstream shielding phase-II enzymes, namely, heme oxygenase 1 (HO-1) and NADPH, NADPH quinone dehydrogenase 1 (NQO1) [34]. Similarly, ER possesses antitumor effects mainly by modulating ROS in human keratinocyte cells [35]. An in vivo study showed that ER has a role in modulating the signals to the transient receptor potential cation channel subfamily V member 1 or the vanilloid receptor 1 (TRPV1) [36]. TRPV1 is liable for intense and constant involvement in signal transduction and adjustment in changing environment. ER maintains a safe balance in T lymphocytes, natural killer cells, and nitric oxide (NO) concentration which can help improve level of cellular toxicity [37]. In addition, ER significantly diminishes intracellular ROS and lipid peroxidation and restrains normal mitochondrial function and hence saves human hepatocellular disease cells (HepG2) [38]. ER safeguards hydrogen peroxide- (H_2O_2-) mediated oxidative damages in prostate cancer (PC12) cells by upregulating the expression of Nrf2/HO-1 and β-glutamyl cysteine synthetase pathways [39]. Furthermore, E7G decreases the expression and activation of Nrf2 in cisplatin-induced harmful effects in human mesangial cells [40].

3.7. Antidiabetic Potential. ER secured high glucose- (HG-) initiated oxidative stress in glomerular mesangial cells (MCs) mainly by repression of inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and IL-6 levels via reducing Akt/NF-κB pathway which supposes that ER may add to its defensive impacts against HG incitement [41]. A dietary intake of ER in mice decreases lipogenesis-related qualities, and an improvement in insulin resistance was reported by suppressing hepatic gluconeogenesis, enhanced glucose metabolism, and modulated the synthesis and discharge of incretin hormones, namely, gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [42]. The studies suggested that the PI3K/Akt pathway in high-glucose HepG2 and cultured 3T3-L1 adipocytes was improved by ER treatment [28]. These findings suggest that ER can upregulate glucose uptake by improving insulin production. ER is a novel stimulator of insulin production that can be applied for glucose-utilization by means of cAMP/PKA signaling pathway [43]. Moreover, it improves glucose resistance and upgrades plasma insulin in nondiabetic and diabetic rodents. ER specifically reduces the degree of retinal aggravation and plasma lipid peroxidation in early diabetic rodents through downregulation of retinal TNF-α, intercellular adhesion molecule 1 (ICAM-1), vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (eNOS) and keeps their amount in sub-

basal levels [44]. Green tea having extracts of ER potentially destroys cholesterol levels (LDL), supplemented by concealment of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) and 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCS) levels and upregulation of low density lipoprotein (LDL) receptor levels in the liver [45]. Moreover, ER repressed α-glucosidase that showed its antidiabetic potential [46].

ER is present in lemon as a natural phytochemical and significantly controls obesity and diabetes [44]. ER has been isolated and characterized as a novel insulin stimulator in both *in vitro* and *in vivo* experiments which could be applied as an elite glucose-suppressant action using cAMP/PKA pathway [43]. In addition, in diabetic rodents that were supplemented with ER, it successfully reduced oxidative stress [7]. The treatment with ER may upregulate the outflow of PPARγ2 and the adipocyte-explicit unsaturated fat restricting protein [44]. Besides, ER exposure fundamentally suppressed diabetes associated lipid peroxidation [47]. ER appeared to provide protection to the rodent retinal ganglion cells- (RGC-) 5 from high glucose-induced oxidative stress and avoided cell apoptosis by means of the activation of Nrf2/HO-1 pathway [48].

3.8. Anti-Inflammatory Effects. ER is a characteristic flavonoid that has been accounted for mitigating and being hostile to osteoclastogenic impacts. ER has an impact on provocative reactions in osteoarthritis (OA), where ER lessens the hindrance of cellular functions in IL-1β-exposed chondrocytes. Similarly, ER hindered the outflows of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), expression of prostaglandin E2 (PGE2), and NO by activating IL-1β production. A huge list of inflammatory cytokines and chain of matrix metalloproteinases (MMPs) were evoked by IL-1β treatment that was additionally weakened by ER. Moreover, ER pretreatment repressed the expression of inhibitor of κBα (IκBα) and p-p65 levels and upregulated the expression of Nrf2 and HO-1 in IL-1β-treated chondrocytes. The small interference Nrf2 (siNrf2) treatment essentially restrained the expression of Nrf2 and ultimately its downstream phase II enzymes such as HO-1 in chondrocytes. Furthermore, siNrf2 transfection in a similar way nullified the calming impacts of ER in chondrocytes. This showed that ER has mitigating impacts on IL-1β-stimulated chondrocytes, while this impact was interceded by restraining NF-κB by triggering Nrf2/HO-1 antioxidant pathway [49].

ER regulated the levels of the inflammatory cytokines such as TNF-α, IL-6, IL-8, and and IL-1β signaling and upgraded forkhead box proteins O1 (FOXO1) in RA-FLS in rheumatoid joints pain [50]. FOXO1 is an Akt downstream factor and the latter gets phosphorylated promptly by decreasing levels of the former.

ER appeared to secure the retinal ganglion cells (RGCs) from high glucose-induced oxidative damage and cell apoptosis by initiation of the Nrf2/HO-1 antioxidant pathways [48]. ER cures atopic dermatitis (AD) in mice through inactivation of IL-4 and upregulation of serum immunoglobulin E (IgE) protein [17]. In IL-1β-activated chondrocytes, ER eased irritation by repressing NF-κB signaling pathway mainly by activating Nrf2/HO-1 antioxidant pathway [49]. Another outcome indicated that ER improves lipopolysaccharides (LPS)-intervened amyloidogenesis and memory hindrance by restraining the toll like receptor 4, mitogen-initiated protein kinase (MAPK), and PI3K/Akt (phosphatidylinositol 3-kinase/protein kinase B) pathways. On the other hand, ER activates the sirtuin 1 (Sirt1) pathway which impedes the downstream movement of NF-κB signaling cascades [2]. Moreover, ER treatment hindered blood urea nitrogen, creatinine, malondialdehyde (MDA), thiobarbituric corrosive, ROS, TNF-α, and IL-1β levels of cisplatin-induced stress in renal tissues [51]. ER modulates the stressful effects of macrophages through the phosphorylation of p38 MAPK, extracellular signal regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK) [29]. Moreover, ER secures UV-driven cytotoxicity in keratinocytes by regulating the phosphatase-subordinate keratinocyte signaling pathways p38 MAPK and Akt pathways [29].

3.9. Immunomodulatory Effects of ER. Macrophages are a significant component in intrinsic immune system and work essentially as phagocytic cells and pose effects via phagocytosing pathogenic creatures [52]. Likewise, enacted macrophages produce enormous measures for NO from arginine and oxygen by different nitric oxide synthases (NOS) as well as decreasing the inorganic nitrates [53]. NO production in the cells is poisonous to microbes and other human microorganisms. Extreme expression of NO responds to superoxide to give rise to peroxynitrites and later is a potent and toxic oxidant by producing a chain of nitrates which can harm a wide variety of proteins, co-factors, enzymes, and biomolecules (DNA and proteins) in cells. Many natural drugs, metabolic products, and ultraviolet radiations are powerful inducers of macrophage NO creation [54]. Investigation demonstrated that ER hindered NO production by peritoneal macrophages. Additionally, ER downregulated NO development [55]. Additionally, ER diminished the NO generation in LPS-treated Raw 264.7 macrophages [29]. The presence of OH bunches at positions 5, 7, 3′, and 4′ along with the twofold bond in the B ring of ER makes it engaged with the high mitigating impact of flavonoids [56]. The presence of two hydroxyl bunches at the 3′ and 4′ places of the B ring of ER could clarify its mitigating movement. Macrophages complete their vague guard work at the end phase of the phagocytic cycle by initiating the lysosomal phosphatase in the cytosolic vesicles [57]. The movement of lysosomal catalysts like basic phosphatase expanded extraordinarily during inflammation cycle and along these lines caused a great damage to the tissues [58]. The current examination demonstrated that ER diminished lysosomal phosphatase activity, uncovering its curative properties.

3.10. Cytoprotective Effects on Kidneys. The defensive impacts of ER on cisplatin- (CP-) induced kidney injury are well illustrated. CP-mediated kidney injury model was built up by using 20 mg/kg of CP. The outcomes indicated that treatment of ER restrained the creation of blood urea nitrogen (BUN), creatinine, MDA, TBARS, ROS, just as the creation of TNF-α, and IL-1β in kidney tissues initiated by CP. ER additionally upregulated the expression of SOD, CAT, and GSH-PX diminished by CP. Moreover, ER was found to up-control the activation of Nrf2/HO-1 and hinder CP-modulated NF-κB activation in the kidneys. Taking the above together, ER showed a protective role against CP-incited kidney injury mainly by activating Nrf2 antioxidant and repressing NF-κB activation [51].

3.11. Hepatoprotective Effects. Liver is an important organ that significantly removes numerous harmful metabolites [59]. The fundamental components of As_2O_3-prompted liver injury have not been yet fully demonstrated. Nonetheless, recent studies showed that oxidative stress generated by As_2O_3 is the sole cause of liver injury [60–62]. Huge literature reported that anticancer agents had remedial effects against arsenic-induced tissue injuries [63,64]. ER has been accounted for to have anticancer properties [36]; moreover, ER leaves cytoprotective effects on As_2O_3-induced liver injury by lessening arsenic mediated obsessive changes in liver tissues. Serum ALT and AST were utilized as biochemical marker of hepatic injury [65]. Moreover, ER hindered As_2O_3-initiated ALT and AST creation and these outcomes lead to proposing that ER displayed extensive defensive impacts against As_2O_3-instigated liver injury.

Arsenic presentation shows oxidative stress by initiating the creation of ROS in liver tissues [61]. The creation of ROS successfully explained following arsenic exposures. Malondialdehyde (MDA) is a noteworthy lipid peroxidation product that multiplies during oxidative stress [66], and it is suggested to utilize ER to screen its efficacy against oxidative stress [67]. The higher levels of MDA have been reported in the liver cells following arsenic exposure. But the treatment of ER surprisingly diminished As_2O_3-generated MDA and ROS production. Besides, the hindrance of SOD, GPX, and CAT upregulation by arsenic was suppressed by ER mainly by scavenging the free radicals and ultimately reducing oxidative stress and liver damage. Nrf2 is an essential leucine zipper protein factor that is accounted for to play a basic role in providing the protection to cancer cells [68]. Activation of Nrf2 prompts the outflow of HO-1, which is a cytoprotective phase-II enzyme for significant heme catabolism [69]. The studies indicated that Nrf2 can be utilized as a successful sub-nuclear factor to neutralize As-mediated harmful effects [70]. Furthermore, ER upregulates the activation of Nrf2 and ultimately HO-1 expression prompted by As_2O_3 exposures.

3.12. Effect of ER on Lungs. Lung cancer is one of the deadly cancers around the globe with hundreds of thousands of patients diagnosed annually [71]. Development of drug resistance has made cancer treatment quite difficult. Therefore natural products are known to have multiple targets and are able to suppress or activate complicated signaling pathways to inhibit tumorigenesis [72]. ER showed potential growth-inhibiting activity against human lung A549 cancer cells but least or no toxicity to the normal human lung cells [21]. ER instigates apoptosis in a focused but highly coordinated way. Mitochondrial outer-layer protrusions (MOPs) are a significant cycle engaged with the apoptotic pathway. It is seen that ER decreases the expression of matrix metalloproteinases (MMPs) and shows a fixation subordinate. ER may potentiate apoptosis by combining intracellular decrease of MMPs expression. It has been accounted for that numerous antitumor drugs may target the diseased cells halfway by causing decrease in MMPs expression [73]. Examination of apoptotic cell explained that ER prompts apoptosis and DNA damage in human cellular degradation in the lungs cells [74]. Furthermore, propidium iodide (PI) confirmed the impacts of ER on cell cycle movement, where G2/M cell cycle arrest was seen for a longer time [75]. Also, impacts of ER on Bcl-2/Bax signaling pathway were assessed by immunoblotting that demonstrated that ER pre-treated cells indicated a focused but assisted downregulation of Bcl-2 and upregulation of Bax proteins leading to activate apoptosis (Figure 2). Individuals from the Bcl-2 family proteins, e.g., Bcl-2, Bax, and Bak, are accepted to assume as key controlling factors in the execution of cellular apoptosis, while Bcl-2 family proteins additionally focused to bypass the apoptotic pathway [75,76]. At long last, the impact of ER on the PI3/AKT/mTOR signaling pathway was explored and it was seen that it caused restraint of a key protein of the pathway. These outcomes are fascinating since this objective is viewed as significant malignancy chemotherapy [21].

3.13. Neuroprotective Effect of ER. ER has been accounted for to have neuroprotective impacts by activating the cellular cancer prevention agents that may protect the cells (Figure 3). It has been discovered that ER secures hydrogen peroxide- (H_2O_2-) instigated neurotoxicity in PC12 cells by means of activation of the Nrf2/ARE cancer prevention agent pathways [39]. Thus, amyloid-β (Aβ) 25-35-activated cell demise in primary cultured neurons is incompletely reduced by ER treatment by means of initiation of the Nrf2/ARE signaling pathway [77]. Another *in vivo* and *in vitro* study exhibited that it eases LPS-modulated oxidative stress, neuroinflammation, and synaptic brokenness through the activation of MAPKs, NF-κB/Sirt1, and Nrf2/Keap1 pathways [2]. Here, MAPKs fundamentally control incendiary reactions constrained by NF-κB. Moreover, ER indicated the promising impact

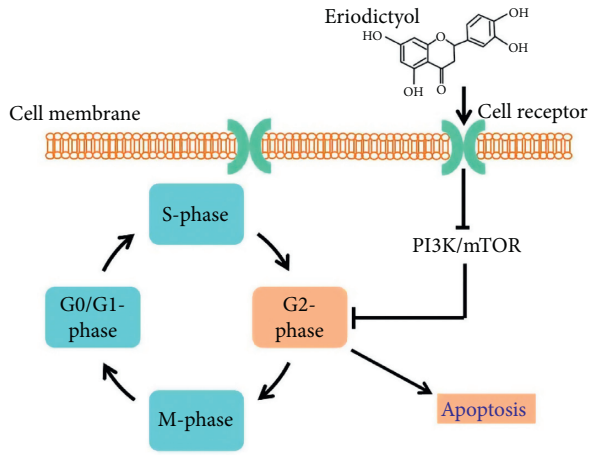

Figure 2: Effect of ER on the cell cycle arrest at G2/M phase, and avoidance of apoptosis. Eriodictyol easily penetrates into the cell via cell receptors and modulates multiple signaling cascades especially PI3K/mTOR that stops the G2-Phase, and hence helps the cells avoid apoptosis.

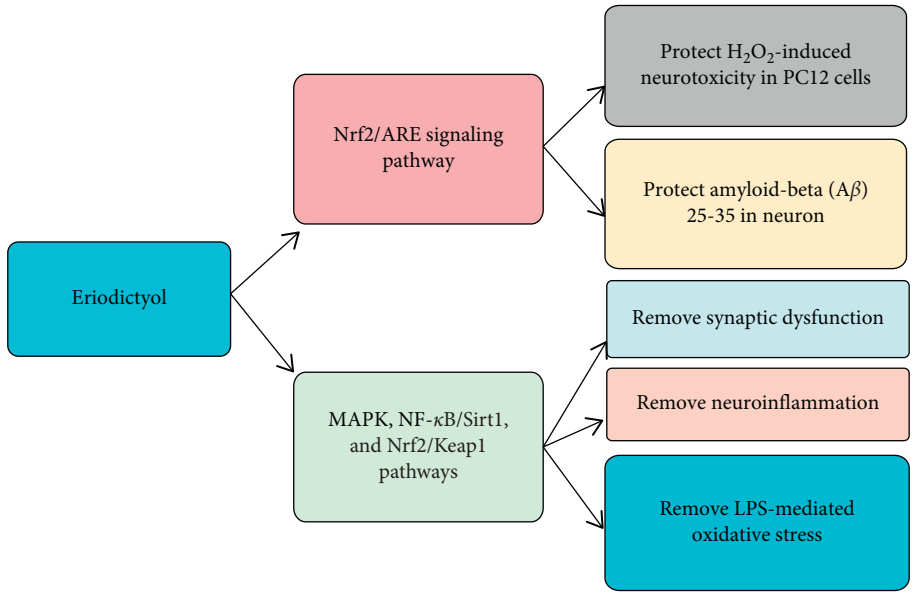

Figure 3: Neuroprotective effects of eriodictyol. Eriodictyol is reported to provide protection via its antioxidant nature by activation of Nrf2/HO-1 cytoprotective pathway. Moreover, Nrf2/HO-1 pathway protects H_2O_2-induced neurotoxicity in prostate cancer cells (PC12), and amyloid-β in neurons, while having anti-inflammatory effects involving MAPK, NF-κB pathway.

of neuroprotection in mice through the guideline of myeloperoxidase, inducible nitric oxide synthase (iNOS), TNF-α, and glial fibrillary acidic protein [78]. The Nrf2/ARE pathway assumes a significant part in the recruitment of phase-II detoxifying compounds and cell reinforcement proteins. In addition, E7G was uncovered to critical insurance against cerebral ischemic injury through the upregulation of the Nrf2/ARE pathways [31].

3.14. Analgesic Activity of ER. The transient receptor potential (TRP) family speaks to a superfamily of particle channels shaped by six transmembrane spaces that are equipped for penetrating cations, fundamentally calcium. These TRPV1 channels are being expressed in every human cell type of all tissues and are linked for the regulation and modulation of multiple cellular functions in different diseases [79,80]; in particular, TRPV1 receptors have great roles in pain treatment [81]. To discover new components that connect with this receptor, the flavonoid ER was tried. ER repressed the known modulator to the TRPV1 receptor and restrained the Ca^{2+} interceded by capsaicin. ER likewise had an antinociceptive impact in the intraplantar and intrathecal capsaicin tests and being antihyperalgesic and hostile to allodynic impacts in the CFA test. Moreover, it saw that ER completely forestalled the oxidative stress instigated by

capsaicin in the spinal line without modifying driving movement or internal heat level [36]. With regard to new compounds that may collaborate with the TRPV1 receptor, we zeroed in on mixes found in therapeutic plants with known antinociceptive impacts. Certain plant extracts in [3H]-RTX needs to explore in detail to figure out which particular active compounds potentially targets and modulates vanilloid site of TRPV1. In view of recent studies, ER dislodges [3H]-RTX officially with more noteworthy strength (around 47 nM) than the old style TRPV1 agonist capsaicin (roughly 3200 nM) [82]. A capsaicin-intervened calcium influx was measured to decide if ER restricting prompts practical balance of the TRPV1 receptor. ER hindered Ca^{2+} inundations with power like that seen in the coupling test and had no impact on Ca^{2+} flooding without capsaicin. This shows that ER goes about as a rival of the TRPV1 receptor and that it restrains Ca^{2+} overflow with more noteworthy power than some old style TRPV1 opponents [83], 5-iodoresineferatoxin (56.7 nM) [84] and capsazepine (7.7 μM) [85].

3.15. Antipyretic and Antinociceptive Activities. The TRPV1 receptor is the most encouraging objective for the improvement of new pain relieving drugs [86]. The vanilloid receptor is appropriated both in the fringe, where it goes about as a sensor of poisonous upgrades, and in the spinal cord, where it partakes in the transmission of torment [87]. Subsequent to confirming that ER goes about as an enemy of the TRPV1 receptor *in vitro*, our following stage was to decide if it could weaken the nociception incited by capsaicin. ER had antinociceptive impacts with high adequacy and intensity when managed either orally or intrathecally. Besides, oral ER administration constricted the nociception activated by intrathecal capsaicin, which proposed that orally regulated ER may arrive at the spinal cord. Hyperthermia is a typical result of TRPV1 rivals, and it was found that ER had no impact on internal heat level in AMG9810-mediated hyperthermia in mice. Organization of AMG517, a simple form of AMG9810, as of late appeared to cause checked and persevering hyperthermia (≈40°C) in people at portions less than those important to evoke a pain-relieving impact (Figure 4). The AMG517-generated hyperthermia has been discovered to be impervious to traditional medicines for fever, e.g., acetaminophen. This hyperthermia was credited to an expansion in fringe vasoconstriction, which diminishes heat misfortune through the skin, expanding the internal heat level [88]. ER has appeared to cause vasorelaxation [89], which could clarify how ER reduces actuate hyperthermia. This information proposed that ER might be a more desirable treatment than many others for the currently known TRPV1 rivals.

Studies showed that capsaicin-incited nociception happens in association with spinal oxidative pressure. Treatment with NAC, a ROS forager, weakened both the oxidative pressure and the nociception prompted by capsaicin. Accordingly, ROS creation has all the earmarks of being a significant instrument of agony acceptance/transmission in mice [90]. The TRPV1 receptor appears to assume a basic part in oxidative stress intervened nociception. The incitement of TRPV1 receptors may instigate oxidative worry in tangible neurons and the spinal rope, and a few oxidants may adjust TRPV1 receptor work [90–92]. Here, we found that ER forestalled the advancement of oxidative worry in the lumbar spinal string. This impact might be expected either to coordinate opposition of the TRPV1 receptor or to have a cancer prevention agent impact. To recognize these other options, we played out the ABTS test and affirmed that ER likewise has a cancer prevention agent impact [92]. Despite the fact that ER has a roughly 38-overlay more noteworthy intensity for threat of the TRPV1 receptor than for cancer prevention agent action, an immediate cell reinforcement impact could contribute, in any event to a limited extent, to its antinociceptive impact [36].

3.16. Miscellaneous Activities. ER could prevent and cure osteolytic diseases characterized by the presence of abnormal osteoclast development mainly due to activity of receptor activator of nuclear factor kappa-B ligand (RANKL) that triggers the differentiation in morphology and functions of osteoclasts via activation of multiple signaling pathways, specifically NF-κB, MAPKs, and Ca^{2+}-dependent signaling, which ultimately activates critical transcriptional factor nuclear factor of activated T-cell cytoplasmic 1 (NFATc1).

Furthermore, it hinders c-Fos and NFATc1 expression, with reduced activation of osteoclast specific genes, namely, cathepsin K, Vacuolar-type H + -ATPase d2 subunit, and tartrate-resistant acid phosphatases (TRAcP/Acp5) [93]. In continuation to this, it is also linked with the avoidance and proper treatment of osteolytic harms identified with multidirectional deviantly expanded expanding osteoclast advancements and capacity. Ribosomal S6 kinase-2 (RSK2) and activating transcription factor 1 (ATF1) play a critical role in transforming neoplastic cells and ER suppressed RSK2 kinase activity in epidermal growth factor- (EGF-) mediated neoplastic cell transformations [94].

Recently, an in silico molecular docking study reported that ER has good binding energies and antiviral effects by its cellular multi-target ability against multiple proteins from SARS-CoV-2, especially the angiotensin converting enzyme 2 (ACE2) [95]. Moreover, compared to many already known drugs, ER strongly binds to virus replicating proteins such as SARS-CoV-2 helicase which potentially helps recognize RNA: DNA duplex is a unique feature of viruses, and active sites of SARS-CoV-2 spike glycoprotein C chain having 10 amino acids [95]. Hence, ER appeared to be a novel multi-target molecule for repurposing against SARS-CoV-2.

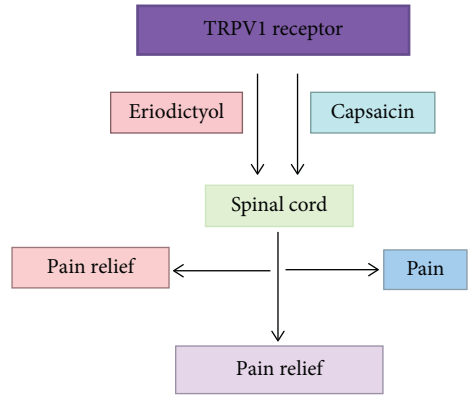

Figure 4: Effect of eriodictyol on pain relief. Eriodictyol mainly hits TRPV1 receptor-mediated activation of the spinal cord and regulates it to relieve pains.

4. Conclusions

All the flavonoids are quite important for therapeutic effects, but ER is the best one for prevention and treating various ailments. ER is extracted from natural sources from various fruits and hence no toxicity or side effects are reported for its use. ER alters the cellular biochemistry and molecular mechanisms for the prevention of onset of multiple diseases. In-depth studies will offer new insights of knowledge and surely will add to another era of flavonoid based therapeutics and nutraceutical options for the treatment of oxidative stress driven diseases.

Data Availability

All data used to support the findings of this study are included within the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Zirong Deng and Sabba Hassan contributed equally to this study.

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Research Article

Study on Network Pharmacological Analysis and Preliminary Validation to Understand the Mechanisms of Plantaginis Semen in Treatment of Gouty Nephropathy

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Plantaginis Semen (PS) has been used to promote diuresis and clear away dampness. Recent reports have shown that PS can be used to treat gouty nephropathy (GN). However, the action and mechanism of PS have not been well defined in treating GN. The present study aimed to define the molecular mechanisms of PS as a potential therapeutic approach to treat GN. A combination of network pharmacology and validation experiments in GN is used to understand the potential mechanism. Information on pharmaceutically active compounds in PS and gene information related to GN was obtained from public databases. The compound target network and protein-protein interaction network were constructed to study the mechanism of action of PS in the treatment of GN. The mechanism of action of PS in the treatment of GN was analyzed via Gene Ontology (GO) biological process annotation and Kyoto Gene and Genomics Encyclopedia (KEGG) pathway enrichment. Validation experiments were performed to verify the core targets. The GN rat model was prepared by the method of combining yeast and adenine. Hematoxylin-eosin (HE) staining was used to observe the morphology of renal tissue in rats. ELISA was applied to detect TGF-β1, TNF-α, and IL-1β levels in renal tissue. The expressions of TGF-β1, TNF-α, and IL-1β were determined using immunohistochemistry. Through the results of network pharmacology, we obtained 9 active components, 118 predicted targets, and 149 GN targets from the public database. Based on the protein-protein interaction (PPI), 26 hub genes for interaction with PS treating for GN were screened, including MMP9, TNF, IL1β, and IL6. The enrichment analysis results showed that the treatment of GN with PS was mainly involved in the TGF-β1 signaling pathway, MAPK signaling pathway, TNF signaling pathway, NF-κB signaling pathway, and PI3K Akt signaling pathway. Validation experiment results showed that PS could reduce the content of urinary protein and UA and deregulate the expression of TGF-β1, TNF-α, and IL-1β in the treatment of GN. The molecular mechanism of PS in the treatment of GN indicated the synergistic features of multicomponent, multitarget, and multipathway of traditional Chinese medicine, which provided an essential scientific basis for further elucidating the mechanism of PS in the treatment of GN.

1. Introduction

Gouty nephropathy (GN) is mainly caused by the deposition of uric acid salt in the blood concentration of the supersaturated state. It is a kind of disease with uric acid stone and interstitial nephritis as the main pathological changes. As the disease progresses, it can eventually lead to kidney failure [1]. It may threaten the life and health of patients in some cases. The GN patients mainly include hyperpimelic middle-aged men and menopausal women, and it is tendency to become younger [2]. The development cycle of GN is relatively slow. In early diagnosis and appropriate treatment, it is essential to improve renal function and stabilize the disease. At present, corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) have been applied to treat GN. However, presently available therapies are not more effective and often

have adverse effects [3, 4]. Literature reported that an increasing number of TCMs have the protective effect of the kidney, and then, increasing attention has been paid to TCM in treatment of nephropathy because it has a good and mild activity.

Plantaginis Semen (PS) is a traditional Chinese herbal medicine with diuretic, anti-inflammatory, and immune regulation effects [5–7]. After extensive research, it was found that PS contains a variety of active components, such as polysaccharides, iridoids, triterpenes, flavonoids, and alkaloids, and many of these ingredients are verified to have certain effects on xanthine oxidase (XOD) activity and uric acid excretion [8, 9]. Under the guidance of traditional Chinese medicine theory, PS is a diuretic drug, and it has the characteristics of mild drug and little side effects. Many prescriptions composed of PS were used for treating GN. Therefore, PS has an important value in the development of GN.

Network pharmacology is based on the theory of system biology and multidirectional pharmacology. It usually uses a complex network to explore the mechanism of action of drugs. It is suitable for studying the relationship between multicomponents and multitargets of traditional Chinese medicine (TCM) [10, 11]. The current study explored the mechanisms of PS for treating GN by applying network pharmacology, to provide credible evidence for the mechanism of PS treating GN.

2. Materials and Methods

2.1. Reagents and Chemicals. Plantaginis Semen was purchased from Tongrentang Chinese Medicine (Beijing, China). Adenine, yeast powder, and allopurinol tablets were purchased from Sigma-Aldrich Co., Ltd. (USA), Chengxin Biotechnology Co., Ltd., and Linfen Qilin Pharmaceutical Co., Ltd., respectively. The detection kits of urine protein, UA, BUN, Cr, and enzyme-linked immune sorbent assay (ELISA) for the measurement of TGF-β1, TNF-α, and IL-1β and CBB were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies TGF-β1, TNF-α, and IL-1β were provided by Beijing Bioss Biotechnology Co., Ltd. (Beijing, China).

2.2. Collection and Screening of Active Components. The TCMSP (http://tcmspw.com/tcmsp.php) is used to search for the useful active components of PS [12]; the key word is "Plantaginis Semen," and the screening condition is "OB $\geq$ 30%, DL $\geq$ 0.18." OB (oral bioavailability) refers to the speed and extent of the drug entering the body circulation after oral absorption [13]. DL (drug-like index) is used to reflect the similarity of specific groups in the compound with known drugs degree [14]. Oral bioavailability and drug-like properties play essential roles in the study of the TCM active ingredient.

2.3. Intersection Analysis of PS and GN-Related Targets. The predicted targets related to PS active ingredient were input into the UniProt database (https://www.uniprot.org/) for screening [15].

The OMIM (http://www.omim.org/) [16] and GeneCards (https://www.genecards.org/) [17] databases were used to collect GN-related disease targets with "Gouty nephropathy" as the keyword. The unnecessary duplicate targets were deleted.

In order to understand the connection between drugs and disease, the PS target and disease target were entered into Venny website (https://bioinfogp.cnb.csic.es/tools/venny/index.html), and the intersection target was obtained.

2.4. Protein-Protein Interaction Data. The intersection target was uploaded to STRING 11.0 (https://string-db.org/) [18], and the irrelevant nodes in the PPI network were hidden. The results were exported in PNG and TSV formats. The TSV results were put into the Cytoscape 3.7.1, and the "network analyzer" plug-in was used to visualize.

2.5. Pathway and Functional Enrichment Analysis. The intersection target was imported into the DAVID database (https://david.ncifcrf.gov/) [19] for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

2.6. Animals. A total of 60 male SD rats, at 10 weeks of age, were purchased from the Animal Experiment Center of Jiamusi University. The animals were housed in polyacrylic cages (five mice per cage) under well-controlled conditions (room temperature of $22 \pm 1^{\circ}$C, relative humidity of $50\% \pm 5\%$, and 12 h : 12 h light/dark cycle) with food and water ad libitum. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jiamusi University, China, and the approval number was JMSU-231.

2.7. Establishment of the GN Rat Model. The GN rat model was prepared by the method of combining yeast and adenine. The suspension of intragastrical gavage (IG) was prepared by 0.5% sodium carboxymethyl cellulose (CMC-Na) solution and adenine. At the same time, the yeast dry powder was mixed into the regular feed of rats, and the daily intake of yeast was controlled to 10 g·kg^{-1}.

Rats were randomly divided into 6 groups ($n = 10$): the control group, model group, positive control group (allopurinol 50 mg·kg^{-1}·d^{-1}),PS high-dose group (1.62 g·kg^{-1}·d^{-1}, PS-HD),PS medium-dose group (0.81 g·kg^{-1}·d^{-1},PS-MD), and PS low-dose group (0.27 g·kg^{-1}·d^{-1},PS-LD). Control group rats were given 0.5% CMC-Na solution in a volume of 100 mg·kg^{-1}. The others were administered with combined adenine (100 mg·kg^{-1}) and 0.5% CMC-Na (100 mg·kg^{-1}) by IG once daily at 8 : 00 a.m. for 28 days. At the beginning of modelling, the rats of each group were separately given PS decoction or allopurinol every afternoon. At 18 consecutive days, the serum samples from the rats' orbital veins were taken for the measurement of contents of UA, Cr, and BUN. The animal model was established successfully if the contents of UA, Cr, and BUN increased significantly. With the prolongation of the modelling time, the rats in the model group showed signs of hair loss, lethargy, arched back, and

weight loss. Each administration group was lighter than the model group, but not as healthy as the normal control group.

2.8. Pharmacological Experiment

2.8.1. Detection of 24 h Urine Protein. The 24 h urine was collected by the metabolic cage method. The 24 h urine protein content was determined by using the urine protein quantitative test box (CBB) in the experiment and on the 7th, 14th, 21st, and 28th days.

2.8.2. Biochemical Parameters. On the 28th day, rats were anaesthetized with 20% urethane. The blood samples were taken from the abdominal aorta, and serum was separated by centrifugation at 3000 r·min^{-1}, 4°C for 10 min. The contents of UA, BUN, and Cr in serum were determined by using an automatic biochemical analyzer.

2.8.3. Renal Weight Index. On the 28th day, the rats were killed immediately, and the kidney index was calculated.

$$\text{Kidney index} = \text{double kidney weight/rat weight} \times 100\%. \quad (1)$$

2.8.4. Detection of Related Enzymes in Serum. According to the instructions of the xanthine oxidase (XOD) kit, the content of XOD in serum was detected by colorimetry.

2.8.5. Histomorphology Observation. The renal tissue was fixed with 10% formaldehyde and, then, embedded in paraffin. Each specimen was cut into 4 μm sections and mounted on APES-coated glass slides. Sections were deparaffinized in xylene, rehydrated in decreasing concentrations of alcohol in water, and then, used for HE staining. The pathological changes of renal tissue were observed under an optical microscope (×400).

2.8.6. Detection of Specific Antibody. Serum TGF-β1, TNF-α, and IL-1β levels were determined by using the ELISA kits.

2.8.7. Semiquantitative Analysis of Immunohistochemical Staining. The expressions of TGF-β1, TNF-α, IL-1β, and IL-6 in renal tissue were detected by immunohistochemistry.

After dewaxing and incubation, the slices were added with citrate buffer solution (PH = 6), heated in a microwave oven, and cooled to room temperature naturally. The first antibody and the second antibody were added successively and incubated at 37°C for color development. They were dehydrated and transparent, sealed, and observed under the optical microscope.

The images were captured with the Motic 3000 (400×). The optical density values of positive reactions in the visual fields were counted by using the Image-Pro Plus 6.0 pathological image analysis system. The integrated optical density (IOD) of positive reactions represented the relative protein expression.

2.9. Statistical Analysis. All values were expressed as mean ± SD. A one-way analysis of variance (ANOVA) was used to detect the statistical significance followed by post hoc Dunn's multiple comparisons test by EXCEL, and $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Screening of PS and GN-Related Targets. Nine active components were obtained from PS, and 118 potential targets of PS were obtained from these nine components (Table 1).

All the targets related to GN were searched in the OMIM and GeneCards database, and 149 GN-related targets were obtained.

In order to clarify the relationship between PS and the GN-related target, 28 intersection targets of PS and GN were obtained (Figure 1).

3.2. Protein-Protein Interaction Analysis. Based on the results of PNG and TSV, the PPI network (Figure 2(a)) contains 28 nodes and 138 edges, and the average number of nodes is 9.36. In order to study the relationship between target proteins, TSV was put into the Cytoscape 3.7.1 for visualization and plotted according to the degree value. The TGF-β1, TNF-α, IL-1β, IL-6, MMP9, and TGF-β1 which had higher degree value may be the key targets for the treatment of GN by active ingredients of PS, as shown in Figure 2(b).

3.3. Enrichment Analysis of Related Pathways and the Biological Process. The DAVID database was used to analyze the GO function analysis and KEGG pathway enrichment analysis of the target genes of PS treating GN. The functional distribution of 58 targets was explored by GO functional analysis, of which 40 entries are related to biological processes (BP), including inflammatory response, response to oxidative stress, and response to hypoxia. Fourteen items are related to molecular function (MF), including cytokine activity and heme binding, and 21 cell components (CC) entries include the extractive matrix and extracellular space (Figure 3(a)). The result indicated that PS might affect the occurrence and development of GN by regulating the abovementioned biological processes.

To determine the relevant signaling pathways involved in the GN effect of PS, we conducted pathway enrichment analysis using KEGG pathways. A total of 28 targets obtained 90 KEGG signaling pathways ($P < 0.01$), including the MAPK signaling pathway, TNF signaling pathway, TGF-β signaling pathway, NF-κB signaling pathway, and PI3K Akt signaling pathway, as shown in Figure 3(b). The results showed that the active target of PS could play a synergistic therapeutic role by regulating multiple pathways.

Table 1: Active components of Plantaginis Semen.

Mol. ID	Active components	OB (%)	DL
MOL001663	(4aS, 6aR, 6aS, 6bR, 8aR, 10R, 12aR, 14bS)-10-hydroxy-2, 2, 6a, 6b, 9, 9, 12a-heptamethyl-1, 3, 4, 5, 6, 6a, 7, 8, 8a, 10, 11, 12, 13, 14b-tetradecahydropicene-4a-carboxylic acid	32.03	0.76
MOL001735	Dinatin	30.97	0.27
MOL000359	Sitosterol	36.91	0.75
MOL005869	Daucostero_qt	36.91	0.75
MOL007813	Dihydrotricetin	58.12	0.28
MOL007819	Hypolaetin	33.24	0.28
MOL007835	Orobanchoside_qt	55.99	0.82
MOL007836	Plantaginin_qt	54.04	0.24
MOL000098	Quercetin	46.43	0.28

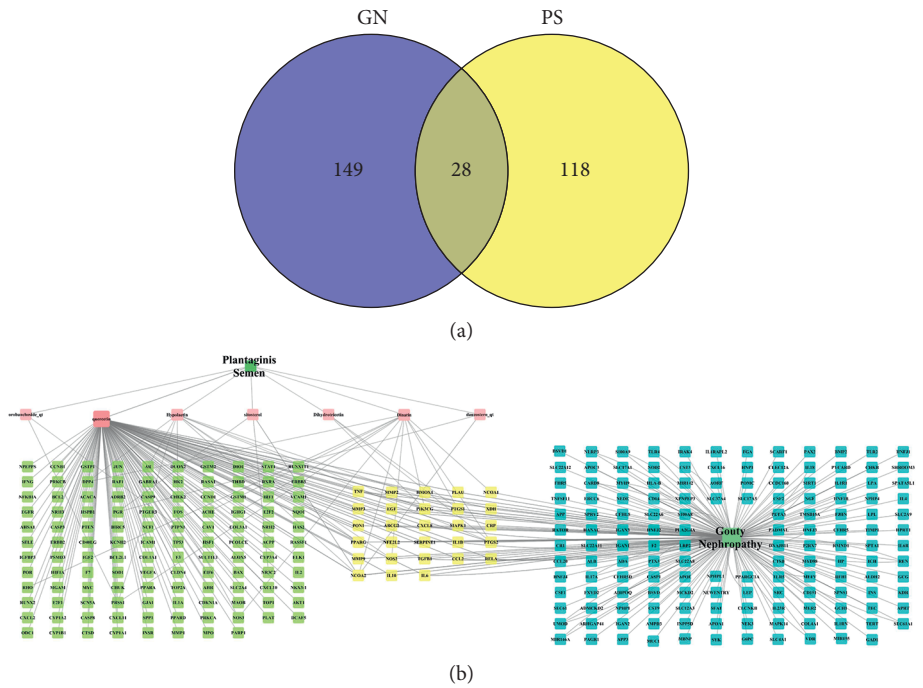


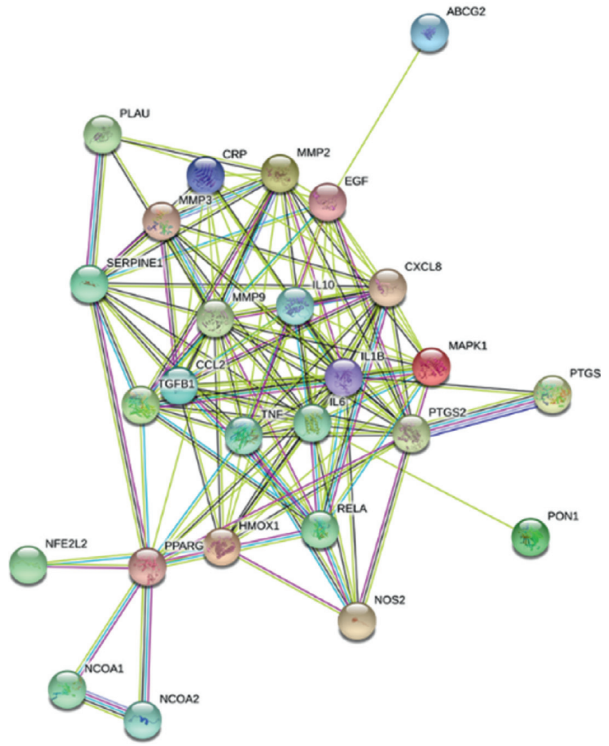
Figure 1: (a) Venn diagram of PS and GN-related targets. (b) PS active compound target-GN-related targets network.

3.4. The Effect on 24 h Urine Protein. There was no significant difference in 24 h urine protein among all groups ($P > 0.05$) on the 7th day. The 24 h urinary protein in the model group rats had increased ($P < 0.01$) compared with that in the control group rats on the 14th and 28th day; the 24 h urinary protein in the PS-HD and PS-MD group rats was decreased ($P < 0.05$), compared with that in the model group rats, as shown in Figure 4. These results indicate that PS can reduce the 24 h urinary protein content of GN rats.

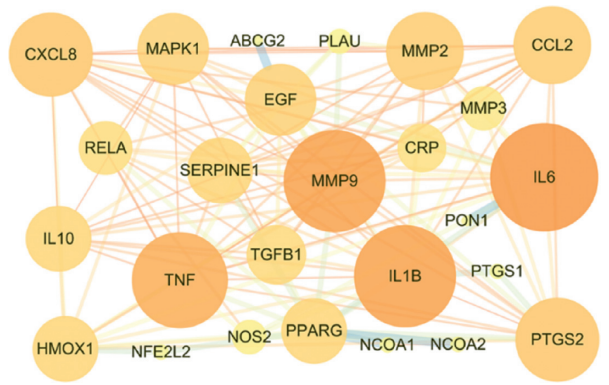
3.5. The Effect on Serum Biochemical Indexes. Compared with the control group, the levels of the UA, BUN, and Cr were increased ($P < 0.01$) in the model group. Compared with the model group, the levels of UA and BUN in the allopurinol group and PS-HD group rats were decreased ($P < 0.01$). The level of UA in the PS-MD group was decreased ($P < 0.01$), and the level of BUN was decreased ($P < 0.05$). Simultaneously, the level of Cr in the allopurinol group, PS-HD, and PS-MD group was decreased ($P < 0.05$), as shown in Figure 5. The results showed that PS could reduce the levels of UA, BUN, and Cr in the serum of GN rats.

3.6. The Effect on the Kidney Weight Index. The kidney weight index in the model group had increased ($P < 0.01$) compared with the control group. Compared with the model group, the kidney weight index of the PS-MD group was decreased ($P < 0.05$); the kidney weight index of the positive group and PS-HD group was decreased ($P < 0.01$), as shown in Figure 6. The results showed that PS could reduce the kidney weight index of GN rats.

3.7. The Effect on XOD in Serum of GN Rats. Compared with the control group, the content of XOD in serum of the model group was increased ($P < 0.01$). Compared with the model



(a)



(b)

Figure 2: PPI network of intersection targets.

group, the content of XOD in the PS-HD group was decreased ($P < 0.05$), as shown in Figure 7. The results showed that PS could reduce XOD in serum of GN rats.

3.8. The Effect on Renal Morphology of GN Rats. In the control group, the renal tissue structure and glomerular morphology were normal; the renal tubular epithelial cells were arranged orderly and uniform in size, without inflammatory cell infiltration and pathological changes.

Compared with the control group, there were more significant pathological changes in the renal tissue of the model group, the number of glomeruli decreased, the vascular atrophy or even disappeared, the edema of renal tubular epithelial cells were prominent, and noticeable yellow-brown urate crystals were visible. There were a large number of inflammatory cells infiltrated in the renal interstitium, which can lead to renal interstitial fibrosis (Figure 8).

Compared with the model group, the pathological changes of renal tissue in each treatment group were alleviated to some extent, the yellow-brown urate crystals of renal tubules were reduced, inflammatory cell infiltration was less, and the degree of renal interstitial fibrosis was slight. The ameliorating effect of the PS-HD group and allopurinol group was significant (Figure 8).

3.9. The Effects on Potential Targets. Compared with the control group, the levels of TGF-β1, TNF-α, and IL-1β in the model group were increased ($P < 0.01$). Compared with the model group, the levels of TGF-β1and IL-1β in the serum of

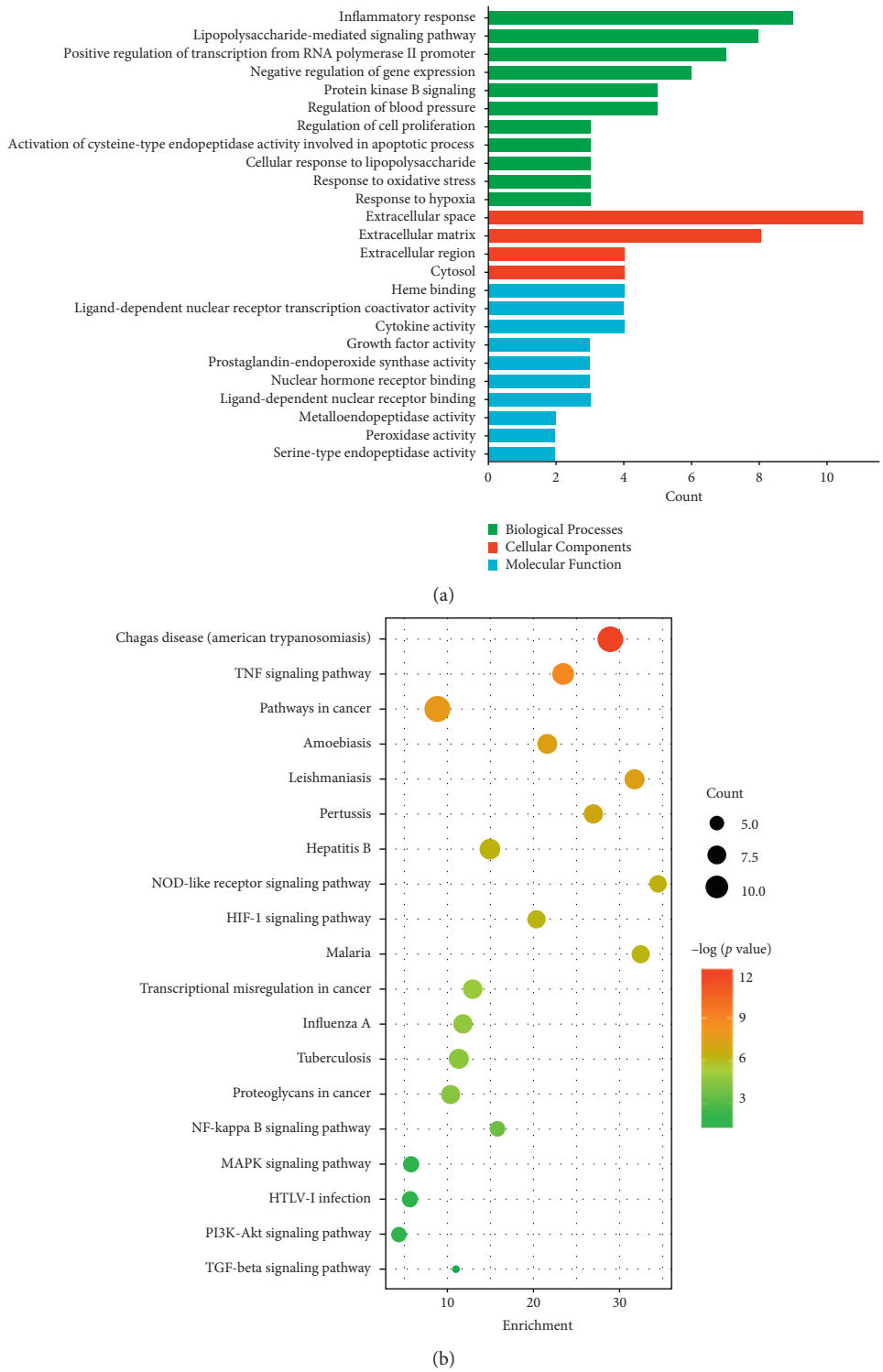


Figure 3: (a) GO enrichment analysis of key targets; (b) KEGG pathways enrichment analysis of key targets.

PS-HD and PS-MD group rats were decreased ($P < 0.01$), and the level of TNF-α was decreased ($P < 0.05$), as shown in Figure 9. The results showed that PS could reduce the expression of TGF-β1, TNF-α, and IL-1β in the serum of GN rats.

3.10. Immunohistochemistry and Semiquantitative Analysis of Renal Tissue. Compared with the control group, the IOD of TGF-β1, TNF-α, and IL-1β in the model group rats was increased ($P < 0.01$). Compared with the model group, the IOD of TGF-β1, TNF-α, and IL-1β in the positive group, PS-

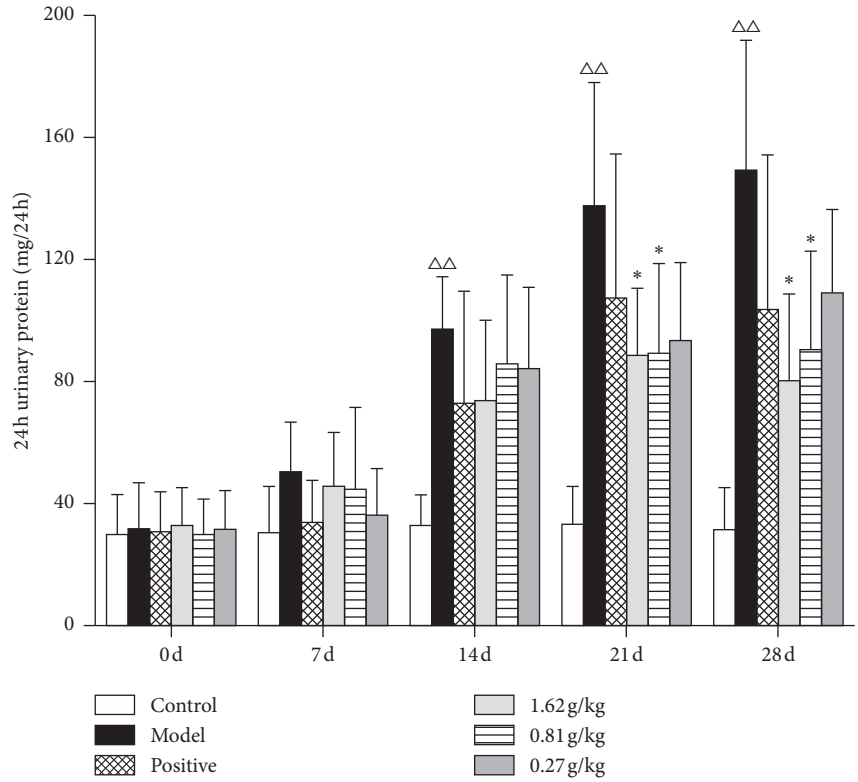


Figure 4: The effect on 24 h urinary protein in GN rats. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the model group.

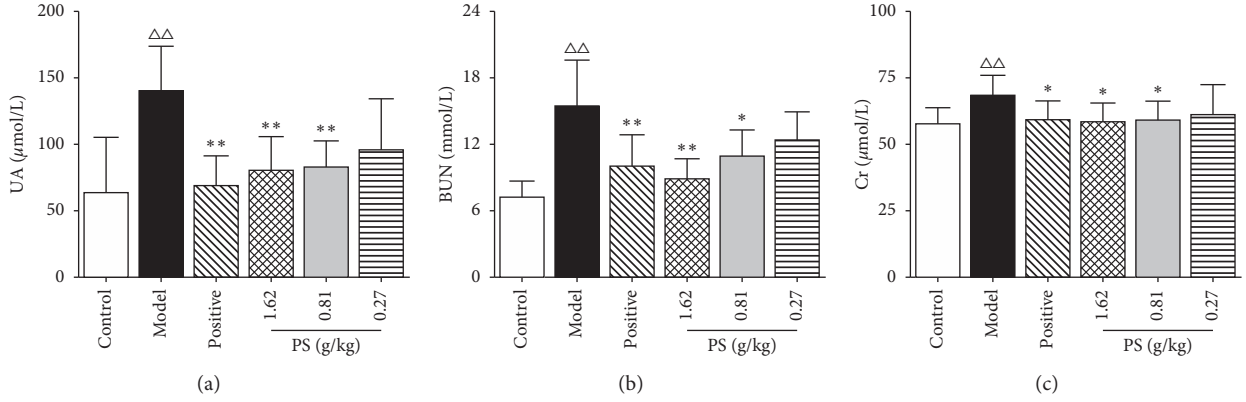


Figure 5: Effect on serum biochemical indexes in GN rats.

HD, and PS-MD group was decreased ($P < 0.01$), as shown in Figure 10. The results showed that PS could significantly inhibit the expression of TGF-β1, TNF-α, and IL-1β protein in renal tissue of GN rats.

4. Discussion

The pathogenesis of GN is usually due to the disorder of purine metabolism, which generates very high production of uric acid or excessively little excretion of uric acid through the kidney, and further leads to the deposition of MSU crystals in the renal tubules and interstitium, ultimately resulting in tubular epithelial cell necrosis, tubular atrophy, and renal interstitial fibrosis [20, 21]. MSU crystal is considered to be one of the critical inducing factors of gout inflammation. It can activate macrophages, cause the production of inflammatory factors such as TGF-β1, TNF-α, IL-6, IL-1β, and oxidative stress, and finally, lead to tissue fibrosis [22–24]. Therefore, MSU crystal activation of inflammatory factors may lead to the development of GN.

According to the results of network pharmacology active components, quercetin, sitosterol, dinatin, dihydrotricetin, and hypolaetin are contained in PS. Quercetin has anti-inflammatory, oxidative stress, and immune regulation effects. It can reverse the deposition of the extracellular matrix of the renal tubular epithelium by inhibiting the expression

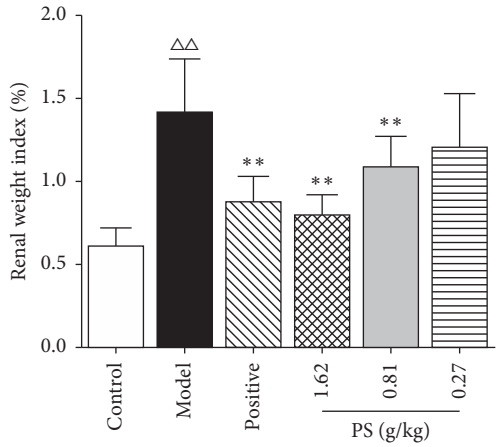


Figure 6: Effect on the kidney weight index in GN rats.

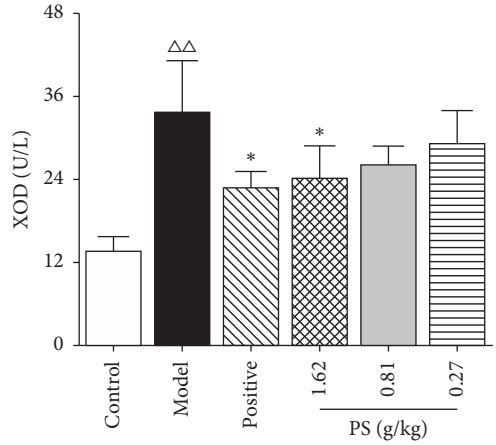


Figure 7: Effect on XOD in GN rats.

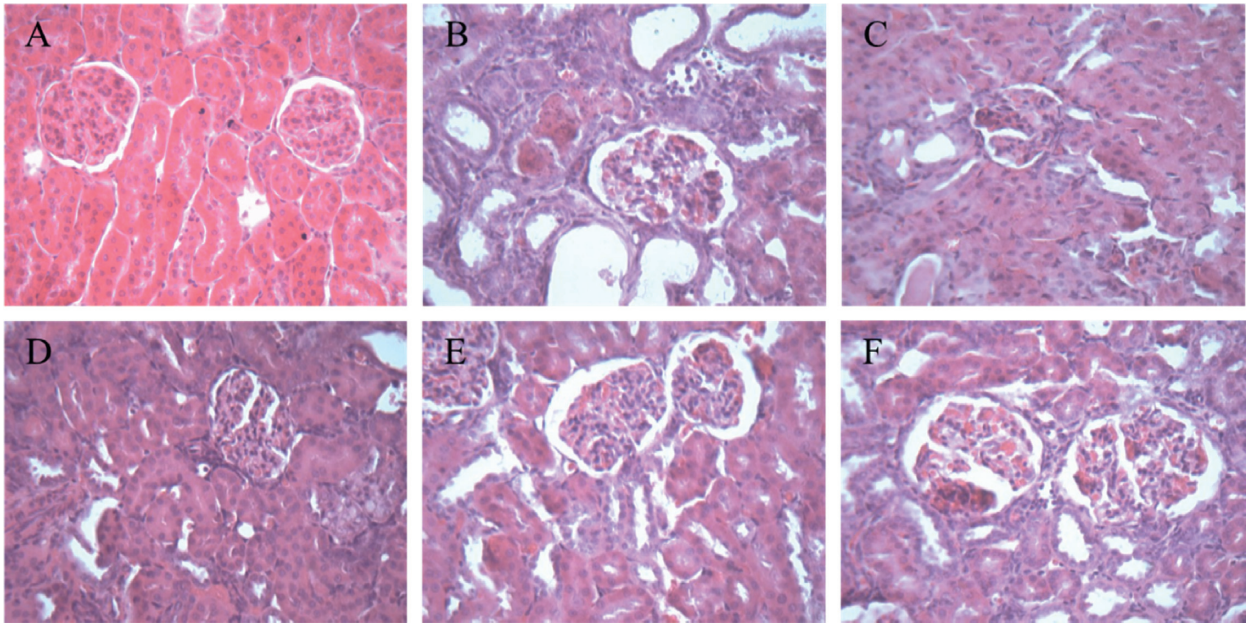


Figure 8: Renal histopathology HE staining (×400). (a) Control. (b) Model. (c) Positive. (d) PS-HD group. (e) PS-MD group. (f) PS-LD group.

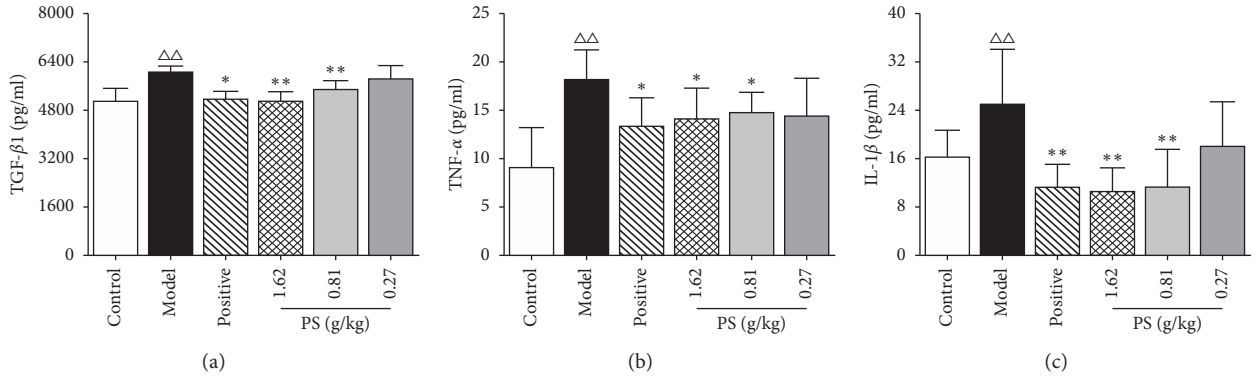


Figure 9: Effect on the expression of inflammatory cytokines in GN rats.

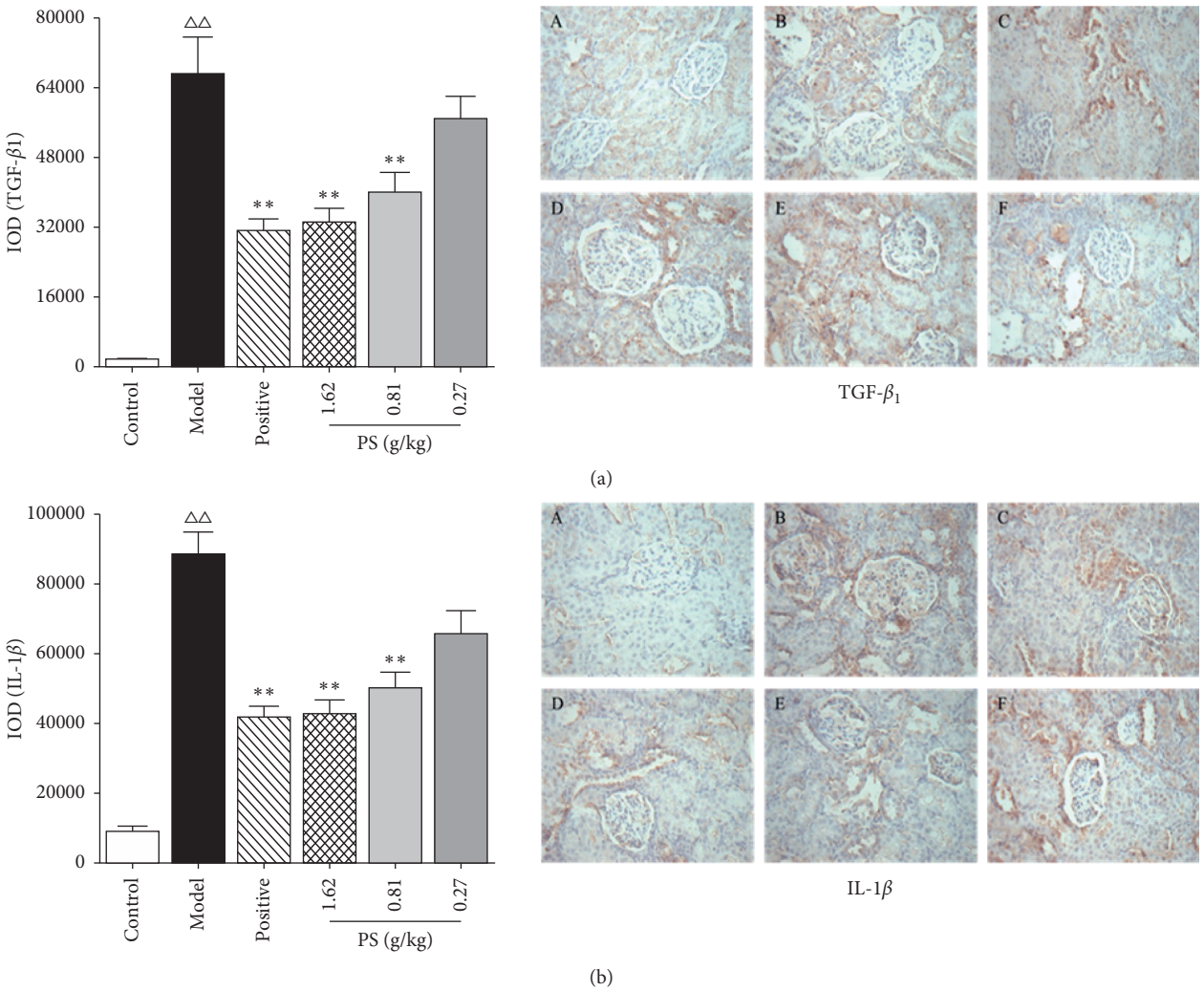


Figure 10: Continued.

of the p38MAPK signaling pathway, thus preventing the significant role in GN activated by MSU [28–30]. TGF-β1

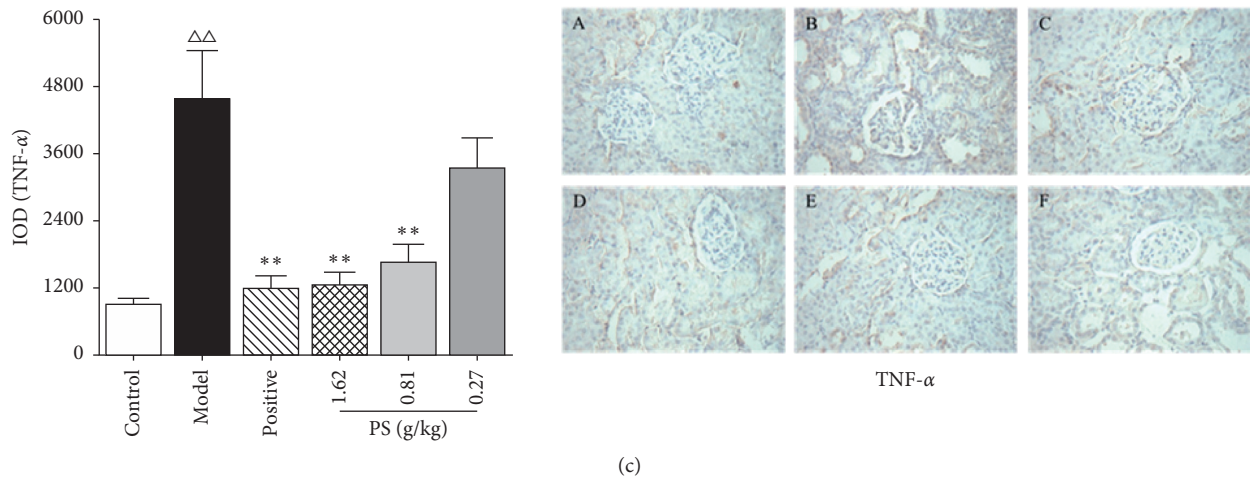


Figure 10: Effect on the expression of TGF-β1, TNF-α, and IL-1β in GN rats. (A) Control. (B) Model. (C) Positive. (D) PS-HD group. (E) PS-MD group. (F) PS-LD group.

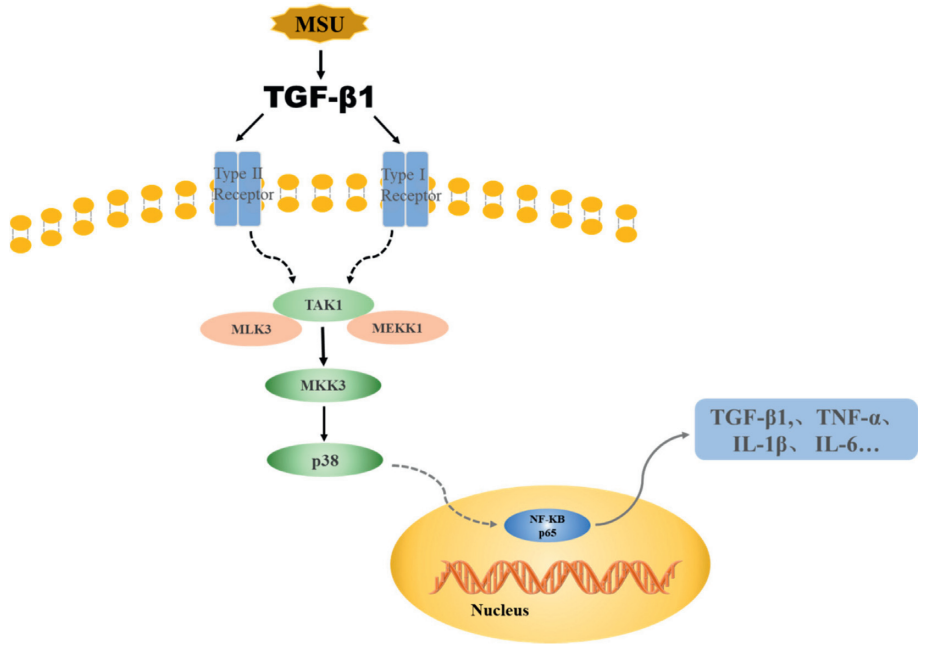


Figure 11: Overview of the potential mechanisms underlying the protective effects of PS on MSU-induced GN.

occurrence and development of renal fibrosis. Sitosterol has an anti-inflammatory effect similar to the hormone, which can block the release of inflammatory factors TNF-α and IL-6 and participate in the regulation of the NF-κB signaling pathway. Dinatin has anti-inflammatory, antioxidant, antitumor, and other pharmacological activities, which can inhibit the expression of NLRP3 inflammatory bodies [25–27].

According to the experimental results of KEGG and PPI in network pharmacology and literature review, the TGF-β1/p38MAPK/NF-κB signaling pathway plays a is a multifunctional cytokine, which can activate the inflammatory signal pathway, promote the infiltration of inflammatory cells, and produce inflammatory factors, thus participating in the process of renal interstitial fibrosis. It is the key cytokine of renal interstitial fibrosis [31, 32]. The p38MAPK is an activated kinase, involving oxidative stress and inflammatory reaction, and can activate the downstream important signal factor NF-κB [33, 34]. After the deposition of MSU crystals in the renal tubules or interstitium, TGF-β1 is stimulated to oversecrete, thus activating TAK1, promoting MKK kinase to

activate p38MAPK phosphorylation. After being phosphorylated, p38MAPK can induce the high expression of the downstream NF-κB signal pathway and, finally, promote the activation of inflammatory factors such as TNF-α, IL-1β, IL-6, and TGF-β1 and aggravate renal interstitial fibrosis and renal tissue inflammatory damage [35, 36], so inhibiting TGF-β1 may be the key of treating GN, as shown in Figure 11.

In order to verify the experimental results of network pharmacology, we used yeast and adenine to prepare the GN rat model and fed PS decoction for pharmacological verification. Gout nephropathy is usually characterized by increased serum UA and urinary protein secretion that can aggravate glomerulosclerosis and renal interstitial fibrosis. Therefore, the determination of UA and urinary protein is of great significance in the diagnosis of GN. The results of pharmacological experiments illustrate that PS could reduce the contents of urinary protein, UA, BUN, and Cr and activity of XOD. Uric acid was the product of purine metabolism, and XOD was the main enzyme of purine compounds metabolism in vivo. UA, BUN, and Cr are the main indicators of renal function, which reflect the uric acid content, glomerular filtration, and renal tubular reabsorption function. When renal parenchymal damage occurs, UA, BUN, and Cr levels increased. Therefore, PS may reduce the level of uric acid by mediating the activity of XOD, accelerate the increase of uric acid in the body, and finally, reduce the damage of renal tissue. The TGF-β1/p38MAPK/NF-κB signaling pathway predicted was verified. Many inflammatory factors play a critical role in renal diseases, such as TGF-β1, TNF-α, and IL-1β; the activation and proliferation of intrinsic cells were resulted by them, and then, the occurrence and progression of renal diseases is aggravated. The results of pharmacological experiments showed that PS can decreased the contents of TGF-β1, TNF-α, and IL-1β in GN rats. It was preliminarily confirmed that PS could regulate the TGF-β1/p38MAPK/NF-κB pathway and improve GN injury, which was consistent with the results predicted by network pharmacology.

5. Conclusions

In this study, the network pharmacology was used to analyze the mechanism and active ingredients of PS treating GN. The GN-related biological targets were measured by validation experiments. It was preliminarily confirmed that PS could inhibit the release and expression of inflammatory factors such as TGF-β1, TNF-α, and IL-1β by regulating the TGF-β1/p38MAPK/NF-κB signaling pathway, to improve renal tissue injury, inflammatory factor infiltration, and renal fibrosis in GN rats. Due to the complexity of the renal fibrosis mechanism, this study aims to lay a foundation for further study of its mechanism.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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