Article

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Loganetin Inhibits Lymphoma Cell Proliferation and Promotes Apoptosis via Inactivating the Wnt/β-Catenin Pathway

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Background: Loganein, the primary active ingredient of Cornus officinalis, has been recognized for its anti-tumor effects in many cancer types, exerting an inhibitory effect on the Wnt/β-catenin pathway. However, its precise impact and underlying mechanism in lymphoma progression are still unclear. This study aimed to investigate whether loganetin regulated lymphoma progression through the Wnt/β-catenin pathway.

Methods: To explore the regulatory impact of loganetin on lymphoma progression, we divided lymphoma cells (Jurkat) into three groups: the Control group (treated with 0 µmol/L loganetin), the Loganetin group (treated with 40, 80, 160 µmol/L loganetin), and the Loganetin+LiCl group (treated with 20 mM Wnt/β-catenin signal activator LiCl and 160 µmol/L loganetin). Subsequently, we assessed Jurkat cell viability, cell cycle, and apoptosis rate to reveal the effect of loganetin and Wnt/β-catenin pathway activator on lymphoma cell growth using cell counting kit-8 (CCK-8) assay, TdT-mediated dUTP Nick-End Labeling (TUNEL) staining assay, and flow cytometry. Moreover, the protein levels of CyclinD1, P21, C-Caspase-3, β-catenin, and c-myc were examined employing RT-qPCR and western blot analysis.

Results: With the increasing of loganetin concentration, Jurkat cell viability, CyclinD1, Bcl-2, β-catenin, and c-myc levels were gradually decreased ($p < 0.05$), while the G0/G1 ratio, P21 level, cell apoptosis rate, TUNEL positive cell rate, as well as the levels of Fas, FASL, Bax and C-Caspase-3/total-caspase 3 were gradually improved ($p < 0.05$). Compared to the Loganetin group, Jurkat cell viability, CyclinD1, Bcl-2, β-catenin and c-myc levels were enhanced ($p < 0.05$), while the G0/G1 ratio, P21 level, cell apoptosis rate, TUNEL positive cell rate, Fas, FASL, Bax and C-Caspase-3/total-caspase 3 levels were reduced in the Loganetin+LiCl group ($p < 0.05$).

Conclusions: Loganetin inactivated the Wnt/β-catenin pathway to restrain lymphoma cell proliferation and promote apoptosis.

Keywords: loganetin; lymphoma; Wnt/β-catenin; apoptosis; proliferation

Introduction

Lymphoma is the most common malignant tumor in China [1,2], with the new cases of Hodgkin lymphoma and non-Hodgkin lymphoma reaching 6984 and 97,788, respectively, according to the WHO GLOBOCAN 2020 data [3]. Current treatment approaches including chemotherapy, local radiotherapy, and hematopoietic stem cell transplantation are insufficient in addressing the demands [4,5]. Therefore, identifying novel and effective therapeutic drugs is necessary to improve the survival rate of lymphoma patients.

Traditional Chinese medicine (TCM) has been used for treating various human diseases [6,7]. Cornus officinalis, a widely used TCM in China, is rich in nutrients and functional components [8,9]. Among the TCM, Loganein, an iridoid glycoside compound, serves as the main active ingredient in Cornus officinalis, demonstrating significant anti-tumor and anti-kidney injury effects [10,11]. However, the effect and mechanism of loganetin on lymphoma progression are unknown.

The Wnt/β-catenin pathway, a classical Wnt signaling cascade, exhibits a wide range of functions such as cell proliferation, apoptosis, and metabolism [12]. In tumor tissues, the Wnt/β-catenin pathway is over-activated, resulting in the malignant phenotype of cancer cells. However, inhibiting this pathway can ameliorate adverse phenotypes [13]. Zhou *et al.* [11] found that loganetin repressed the progression of gastric cancer by inhibiting the Wnt/β-catenin pathway. Therefore, we hypothesized and explored whether loganetin regulates lymphoma progression through the Wnt/β-catenin pathway. This study aimed to provide comprehensive insights into the development of therapeutic options for lymphoma.

Materials and Methods

Cell Culture and Grouping

Lymphoma cells (Jurkat; CBP60520, Cobioer, Nanjing, China) were cultured in RPMI-1640 (11875119, Gibco, Carlsbad, CA, USA) medium supplemented with

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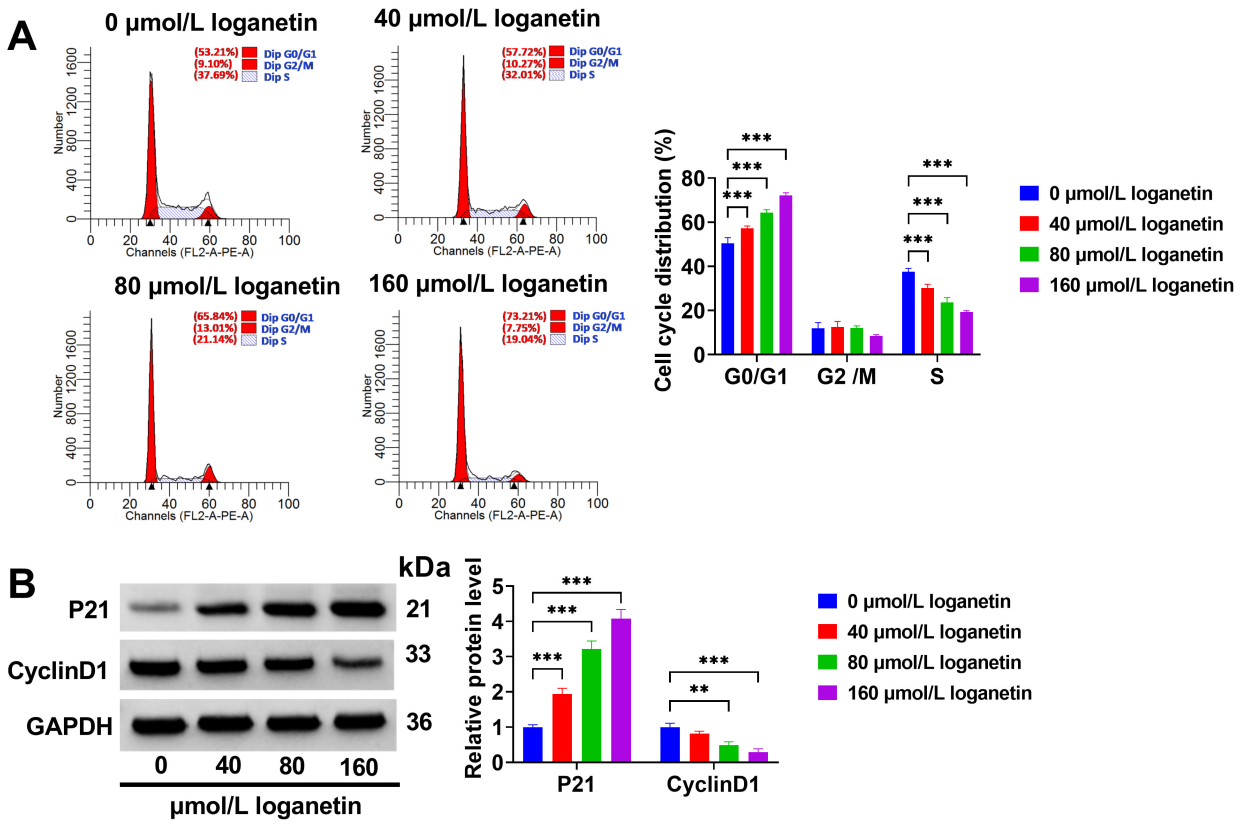

Fig. 1. Effect of loganetin on Jurkat cell cycle. (A) Cell cycle was analyzed using flow cytometry (n = 3). (B) CyclinD1 and P21 protein levels were assessed employing western blot analysis (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$.

1% penicillin/streptomycin and 10% FBS (10099158, Gibco, Carlsbad, CA, USA). The cell culture was examined for contamination using the mycoplasma test and found negative, and was authenticated through STR profiling. The cells were divided into three groups: the Control group (cells treated with 0 μmol/L loganetin), the Loganetin group (cells treated with 40, 80, and 160 μmol/L loganetin), and the Loganetin+LiCl group (cells treated with 20 mM Wnt/β-catenin signal activator LiCl and 160 μmol/L loganetin).

Cell Counting Kit-8 (CCK-8) Assay

Initially, Jurkat cells were seeded into 96-well plates. Subsequently, they were treated with different concentrations including 10, 20, 40, 80, and 160 μmol/L of loganetin (29748-10-5, Mreda, Beijing, China) for 48 hours. The cells without treatment (0 μmol/L) were used as the control group. Furthermore, a group of cells was treated with 160 μmol/L loganetin, either with or without the presence of 20 mM LiCl (L9650, Sigma-Aldrich, St. Louis, MO, USA) for 48 hours. After this, the cells were incubated with CCK-8 solution (CK04, Dojindo, Kumamoto, Japan). Finally, absorbance was assessed at 450 nm using SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, CA, USA) to analyze cell viability.

Flow Cytometry

The treated Jurkat cells were collected, and subsequently fixed with 70% ethanol followed by staining with 0.5% PI solution (C1052, Beyotime, Shanghai, China) to examine the cell cycle. In the next step, the cells underwent staining with Annexin V-FITC (C1062S, Beyotime, Shanghai, China) and PI solutions, and the cell apoptosis rate was evaluated using a flow cytometer (LSRFortessa TM X-20, BD Biosciences, San Diego, CA, USA).

TdT-Mediated dUTP Nick-End Labeling (TUNEL) Staining Assay

The cells were collected and fixed with 4% paraformaldehyde. Subsequently, they were treated with 0.3% Triton X-100 followed by co-incubation with TUNEL reaction solution (C1086, Beyotime, Shanghai, China). Finally, the cells were observed using a fluorescence microscope (SMZ18, Nikon, Tokyo, Japan), and the TUNEL positive cell rate was analyzed through ImageJ software (version 1.8.0, NIH, Bethesda, MD, USA).

Western Blot

Total protein was extracted, separated on 10% SDS-PAGE, and subsequently transferred onto a PVDF membrane. The membrane was incubated overnight with primary antibodies at 4 °C. The following day, the membrane

Table 1. Effect of loganetin on Jurkat cell viability (n = 3).

Groups	Concentrations (μmol/L)	Cell viability (%)
Control	0	100.00 ± 11.23
	10	96.36 ± 11.45
	20	89.75 ± 10.15
Loganetin	40	75.23 ± 6.66*
	80	60.78 ± 5.19*#
	160	42.69 ± 4.83*#&
F		19.871
p		<0.001

Note: *$p < 0.05$ to 0 μmol/L; #$p < 0.05$ to 40 μmol/L; &$p < 0.05$ to 80 μmol/L.

was thoroughly washed and incubated with secondary antibodies for one hour. After this, the protein bands were visualized utilizing an ECL reagent (P0018S, Beyotime, Shanghai, China). The antibodies (Abcam, Cambridge, CA, USA) used in this assay were as follows: anti-CyclinD1 (1:200, ab16663), anti-P21 (1:1000, ab109520), anti-Fas (1:1000, ab82419), anti-FASL (1:1000, ab302905), anti-Bax (1:1000, ab32503), anti-Bcl-2 (1:1000, ab32124), anti-caspase 3 (1:5000, ab32351), anti-β-catenin (1:4000, ab16051), anti-c-myc (1:1000, ab32072), anti-GAPDH (1:2500, ab9485), and secondary antibodies (1:50,000, ab205718). The Gray values of protein bands were analyzed through ImageJ software (version 1.8.0, NIH, Bethesda, MD, USA). The GAPDH was used as an internal control.

Statistical Analysis

Data were statistically analyzed using SPSS software (version 21.0, IBM, Armonk, NY, USA) and were presented as mean ± SD. The differences between the two groups were compared using Student's *t*-test and multiple group comparisons were performed through ANOVA (one-way or two-way) followed by Tukey post-hoc test. A *p*-value < 0.05 was considered significantly significant.

Results

Loganetin Repressed Jurkat Cell Viability

The cell viability remains unchanged after treatment with 10 and 20 μmol/L of loganetin compared to the Control group. However, a gradual decrease was observed in cell viability at 40, 80, and 160 μmol/L loganetin treatments ($p < 0.05$) (Table 1). Therefore, 40, 80, and 160 μmol/L of loganetin were selected for subsequent experiments.

Loganetin Suppressed the Jurkat Cell Cycle

G0/G1 ratio and P21 protein levels were progressively increased ($p < 0.05$), while CyclinD1 protein level was gradually decreased upon treating the cells with 40, 80, and 160 μmol/L of loganetin ($p < 0.05$, Fig. 1). The findings suggest an inhibitory effect of loganetin on the Jurkat cell cycle.

Table 2. Effect of loganetin and LiCl on Jurkat cell viability (n = 3).

Groups	Viability (%)
Loganetin	100.00 ± 7.81
Loganetin+LiCl	167.23 ± 14.69*
t	6.999
p	0.002

Note: Compared to the Loganetin group, *$p < 0.05$.

Loganetin Exposure Induced Jurkat Cell Apoptosis

As shown in Fig. 2, cell apoptosis rate, TUNEL positive cell rate, as well as the levels of Fas, FASL, Bax, and C-Caspase-3/total-caspase 3 were gradually enhanced, whereas Bcl-2 level was gradually reduced in 40, 80, and 160 μmol/L Loganetin-treated groups ($p < 0.05$).

Loganetin Regulated Wnt/β-Catenin Pathway

β-catenin and c-myc protein levels were significantly decreased in Jurkat cells treated with 40, 80, and 160 μmol/L of loganetin ($p < 0.05$, Fig. 3).

Wnt/β-Catenin Pathway was Activated by LiCl in Loganetin-Treated Jurkat Cells

Protein levels of β-catenin and c-myc were substantially higher in the Loganetin+LiCl group compared to the Loganetin group ($p < 0.05$, Fig. 4).

LiCl Regulated Proliferation, Cell Cycle, and Apoptosis in Loganetin-Treated Jurkat Cells

The cellular viability and the expression levels of CyclinD1 and Bcl-2 proteins were substantially increased ($p < 0.05$), while G0/G1 ratio, apoptosis rate, as well as the expression levels of P21, Fas, FASL, Bax, and C-Caspase-3/total-caspase 3 proteins were significantly decreased in the Loganetin+LiCl group ($p < 0.05$, Fig. 5 and Table 2).

Discussion

Loganin, the main component found in Cornus officinalis, which belongs to the iridoid glycosides family, exhibits anti-inflammatory, immune regulation, and hypoglycemic effects [14–16]. Loganetin is a light yellow substance produced from loganin by removing one molecule of glucose [17]. A previous study has shown that loganetin could protect against acute kidney injury induced by rhabdomyolysis [10]. Furthermore, it plays an anti-tumor role in gastric cancer by inhibiting cancer cell proliferation, metastasis, and stem-like properties [11]. In this, we observed that loganetin treatment suppresses lymphoma cell viability, enhances apoptosis, and induces cell cycle arrest, indicating its role in inhibiting lymphoma cell growth.

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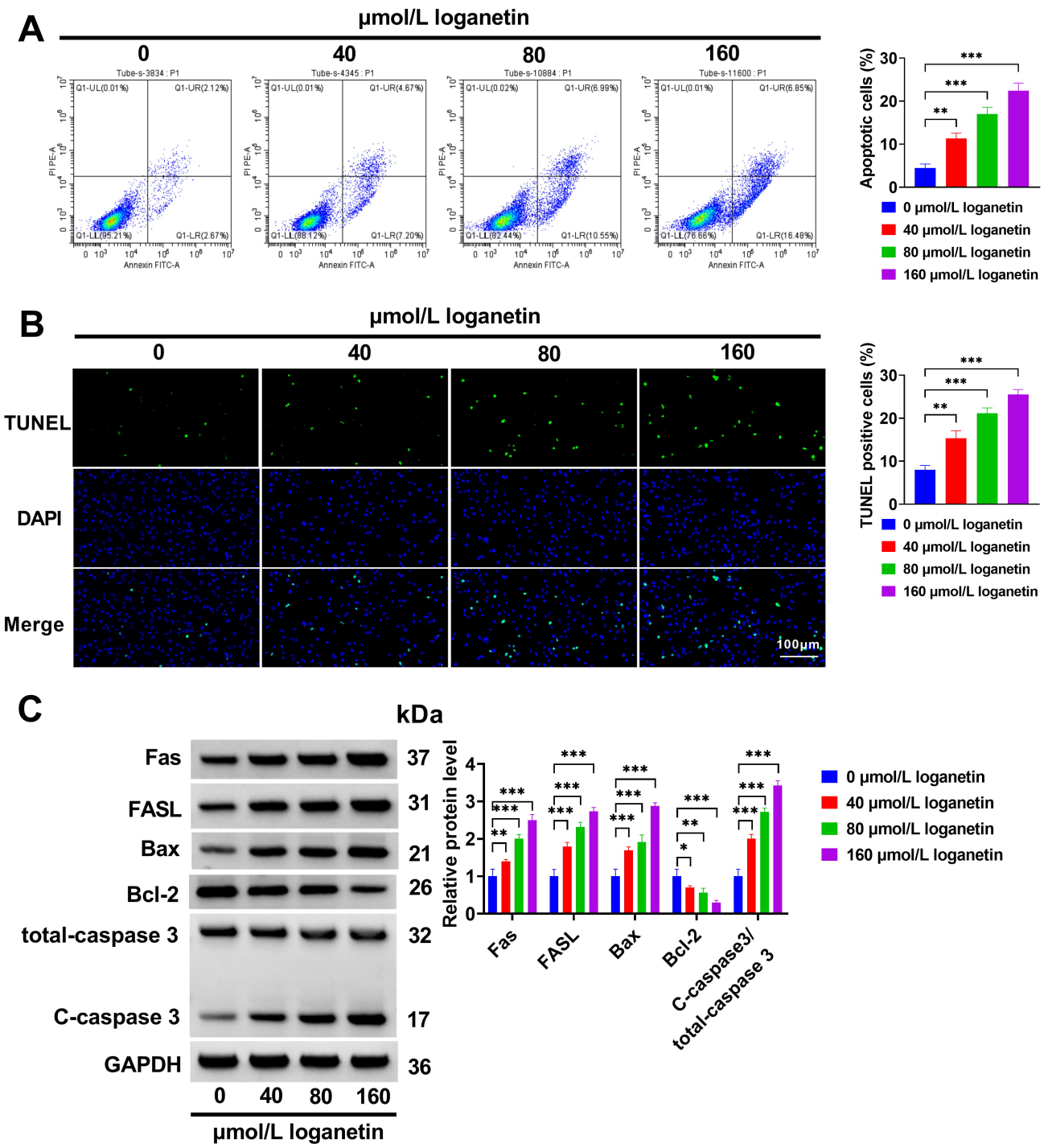

Fig. 2. Effect of loganetin on Jurkat cell apoptosis. (A) Cell apoptosis rate was assessed using flow cytometry (n = 3). (B) TdT-mediated dUTP Nick-End Labeling (TUNEL) positive cell rate was analyzed employing the TUNEL staining method (n = 3). (C) Protein levels were examined through western blot analysis (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Cell proliferation relies on orderly progression of the cell cycle [18,19], with a crucial regulatory point being the transition from G0/G1 to the S phase, regulated by multiple genes [20,21]. CyclinD1, a primary regulator expressed in the G0/G1 phase, promotes entry of the cells to the S phase, thereby inducing cell cycle progression [22,23]. In contrast, P21 acts as an inhibitor of the cell cycle, blocking the cell cycle process and reducing the cell proliferation rate [24,25]. The caspase protein family serves as regulator closely associated with cell apoptosis, and its activation induces cellular apoptotic process [26,27]. Caspase-3 acts as the primary executing factor of apoptosis within the Caspase protein family, and the activation of caspase-3 (C-Caspase-3) is also recognized as a biomarker of apoptosis [28,29]. Our data revealed that CyclinD1 and Bcl-2 levels were significantly decreased, while P21, Fas, FASL, Bax,

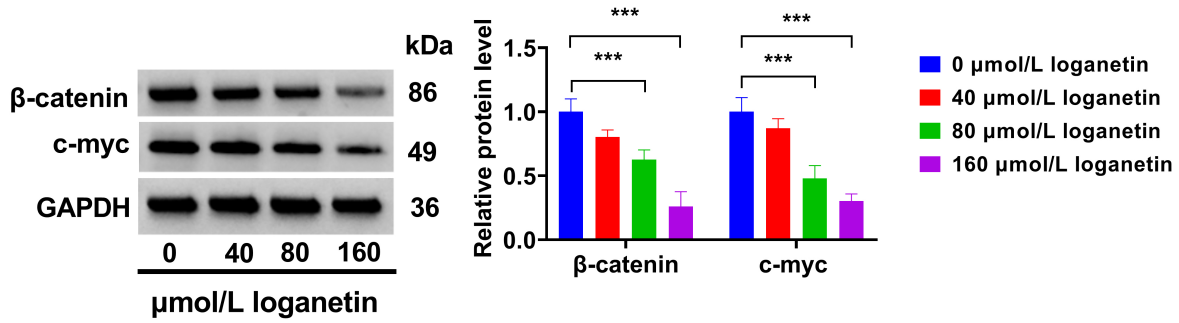

Fig. 3. Effect of loganetin on β-catenin and c-myc protein levels. Protein levels were determined in the Loganetin-treated Jurkat cells using western blot analysis (n = 3). ***$p < 0.001$.

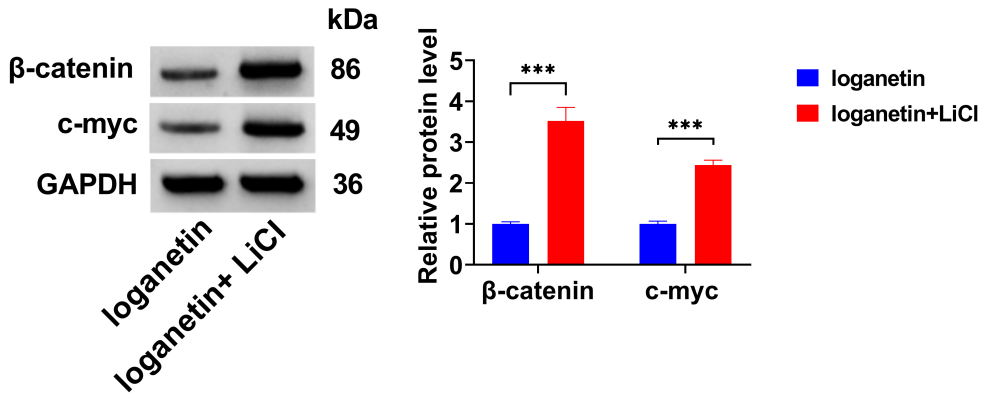

Fig. 4. Effect of Loganetin+LiCl on β-catenin and c-myc protein levels. Protein levels were evaluated in Loganetin+LiCl-treated Jurkat cells using western blot analysis (n = 3). ***$p < 0.001$.

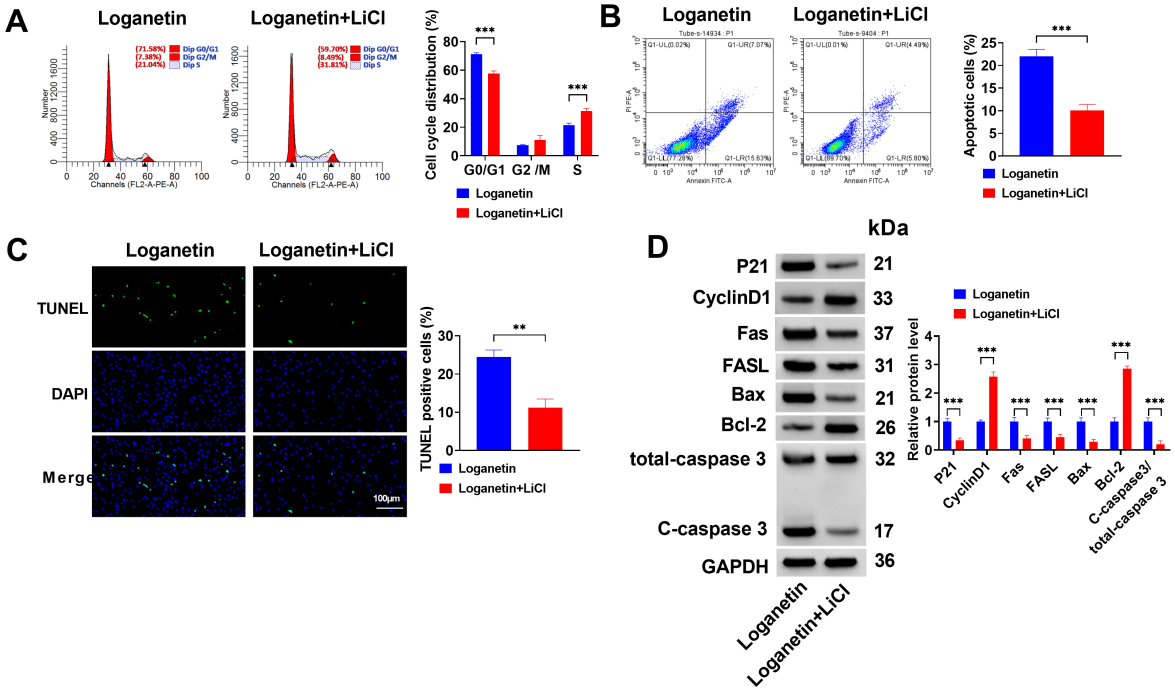

Fig. 5. Effect of loganetin and LiCl on cell apoptosis, C-Caspase-3, CyclinD1, and P21 protein levels. (A,B) Cell cycle and the number of apoptotic cells were assessed using flow cytometry (n = 3). (C) TUNEL positive cell rate was analyzed utilizing TUNEL staining (n = 3). (D) Protein levels were detected using western blot analysis (n = 3). **$p < 0.01$, ***$p < 0.001$.

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and C-Caspase-3/total-caspase 3 levels were increased in the lymphoma cells treated with loganetin. These findings further indicate that loganetin induces both cell cycle arrest and apoptosis in lymphoma cells.

Traditional Chinese medicine mainly exerts its impact by affecting gene expression in tumor cells or altering the transduction of signaling pathways [30,31]. The β-catenin-mediated Wnt pathway is the most classical Wnt pathway, with c-myc serving as a downstream gene [32,33]. The wnt/β-catenin pathway plays a substantial role in human cancer, and it is often over-activated in tumor tissues [34,35]. The Wnt/β-catenin pathway has been found activated in lymphoma cells, and its inactivation was observed to suppress lymphoma cell proliferation and migration [36]. The relevant experimental results revealed that inhibition of β-catenin can induce apoptosis and cell cycle arrest in lymphoma cells [37]. In our study, we confirmed that loganetin reduced β-catenin and c-myc protein levels in lymphoma cells, indicating its inhibitory impact on the Wnt/β-catenin pathway. Moreover, LiCl treatment abolished the regulatory impact of loganetin on lymphoma cell proliferation and apoptosis, providing additional validation that loganetin affects lymphoma cell proliferation and apoptosis through the Wnt/β-catenin pathway.

However, there are some limitations in our study. Presently, we conduct experiments involving cell lines. Due to technical constraints, obtaining primary animal lymphocytes has been unfeasible. Our future studies may involve the extraction of primary T lymphocytes from animals for this purpose. Furthermore, *in vivo* experiments are necessary, and we will explore the effect of loganetin on lymphoma progression using animal experiments. Additionally, the specific targeting mechanism through which loganetin affects lymphoma cell proliferation and apoptosis is yet to be explored. This will be further explored in future studies.

Conclusions

In summary, our data showed that loganetin could inhibit lymphoma cell proliferation and promote apoptosis by inactivating the Wnt/β-catenin pathway. These findings suggest that loganetin may be an effective drug for treating lymphoma.

Availability of Data and Materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Author Contributions

XRY and XJY designed and performed the research study, collected and analyzed the data, and were involved in drafting the manuscript. Both authors have been involved in revising it critically for important intellectual content. Both authors gave final approval of the version to be published. Both authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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