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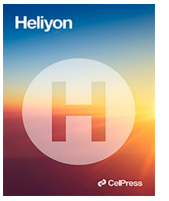

Research article

Huaier inhibits autophagy and promotes apoptosis in T-cell acute lymphoblastic leukemia by down-regulating SIRT1

Xiang Qin [a,1], Xi Chen [a,1], Fan Wang [b], Fangfang Zhong [a], Yan Zeng [a], Wenjun Liu [a,*]

[a] Department of Pediatrics, Children Hematological Oncology and Birth Defects Laboratory, The Affiliated Hospital of Southwest Medical University, Sichuan Clinical Research Center for Birth Defects, Luzhou, Sichuan, 646000, China
[b] Department of Newborn Medicine, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, 646000, China

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ABSTRACT

Objective: Due to the high drug resistance and relapse rate of T-cell acute lymphoblastic leukemia (T-ALL), the prognosis is usually poor. Therefore, there is an urgent need to find safer and more effective therapeutic drugs. Huaier and its preparations, as adjuvant drugs, have been widely used in the treatment of solid tumors and other diseases. However, the application of Huaier in leukemia is rarely reported. In this study, we investigated the anti-tumor effect of Huaier on T- ALL and its underlying mechanism.

Methods: Jurkat and MOLT-4 cells were treated with Huaier. Cell viability was evaluated by CCK-8 assay. The morphological changes of apoptotic cells were observed by Hoechst 33258 staining. Cell apoptosis was analyzed by flow cytometry. The expression levels of related proteins were assessed by Western blot.

Results: The results showed that Huaier significantly inhibited the proliferation of Jurkat and MOLT-4 cells in a dose- and time-dependent manner, with IC_{50} of 2.37 ± 0.10 and 1.93 ± 0.07 mg/mL at 48 h, respectively. Morphological changes and increased number of apoptotic cells were observed by Hoechst 33258 staining and flow cytometry. The apoptosis rates of Jurkat and MOLT-4 cells in 4 mg/mL group were 50.67 ± 1.36 % and 49.97 ± 5.43 %, respectively. Huaier promoted the expression of Cytochrome *c*, Cleaved Caspase-3, Cleaved PARP, p53, LC3-II and p62 proteins, while inhibited the expression of SIRT1, ATG7 and Beclin 1 proteins. Treatment with SRT1720 (SIRT1 agonist) combined with Huaier rescued Huaier-induced apoptosis and increased the expression of autophagy-related proteins.

Conclusion: Huaier inhibits autophagy and promotes apoptosis of T-ALL cells by down-regulating SIRT1, which may be a potential drug for the treatment of T-ALL.

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological disease characterized by the abnormal proliferation of immature T cells in bone marrow and lymphoid tissues [1]. The incidence of T-ALL accounts for approximately 15 % of pediatric ALL cases, whereas it accounts for 25 % of adult ALL cases [2]. To date, although the prognosis of T-ALL patients has been significantly improved due to the application of intensive chemotherapy regimens and allogeneic hematopoietic cell transplantation and other

* Corresponding author.
E-mail address: wenjun_liu@swmu.edu.cn (W. Liu).
[1] These authors contributed equally to this work.

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treatment strategies, there are still 20 % of children and 50 % of adult T-ALL patients with drug resistance and eventually relapse, and the cure rate is less than 30 % [3,4]. In addition, in view of the characteristics of chemotherapy drugs such as large side effects, single target and easy drug resistance, traditional Chinese herbal medicines with low toxicity, multiple targets and low prices have become a new research focus.

Huaier, also known as Trametes robiniophila Murr, is a very important medicinal fungus. Recent studies have shown that Huaier possesses many biological activities, such as anti-tumor and enhancing immune function, and has obvious low toxicity in vitro and in vivo, as well as in clinical studies [5]. Basic studies have shown that Huaier induces immunogenic cell death in triple-negative breast cancer through CircCLASP1/PKR/eIF2α signaling pathway [6], activates ferroptosis by inducing autophagy in pancreatic cancer [7], and inhibits the proliferation of non-small cell lung cancer by targeting epidermal growth factor receptor [8]. Currently, Huaier related preparations on the market include Huaier granules and Huaiqihuang granules. Huaier granules have been widely used as adjuvant drugs for tumors. A propensity score analysis of oncologic outcomes showed that Huaier granules significantly prolonged 5-year overall survival in patients with hepatocellular carcinoma [9]. Huaier granules also improved disease-free survival and overall survival in patients with breast cancer and gastric cancer [10–12]. Huaiqihuang granules reduces the recurrence rate and infection frequency in children with primary nephrotic syndrome [13]. Network pharmacology analysis showed that Huaiqihuang granules could treat immune thrombocytopenia [14]. Although Huaier and its preparations have achieved remarkable efficacy in solid tumors, there is a lack of relevant research in leukemia.

In this study, we investigated the anti-proliferative effect of Huaier on T-ALL cell lines and the underlying mechanism to provide more options for the treatment of leukemia.

2. Materials and methods

2.1. Reagents

Huaier extract was provided by Qidong Gaitianli Medicine Co., Ltd. (Jiangsu, China). Z-VAD-FMK was purchased from MCE (New Jersey, USA). SRT1720 was obtained from Selleck (Houston, USA). Cleaved caspase-3 (#9664, 1:1000), Cleaved PARP (#9546, 1:1000), Cytochrome *c* (#11940, 1:1000), ATG7 (#8558, 1:1000) and LC3 (#12741, 1:1000) were purchased from Cell Signaling Technology (Beverly, MA, USA). SIRT1 (ab110304, 1:2000) and p62 (ab82645, 1:2000) was provided by Abcam (Cambridge, UK). p53 (sc-126, 1:500) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Beclin 1 (11306-1-AP, 1:1000) and GAPDH (10494-1-AP, 1:10000) were provided by Proteintech Group (Wuhan, China). Goat anti-mouse (A0216, 1:2000) and goat anti-rabbit (A0208, 1:2000) antibodies were obtained from Beyotime Biotechnology (Shanghai, China).

2.2. Cell lines and cell culture

Jurkat and MOLT-4 cell lines were purchased from ZhongqiaoXinzhou Biotechnology Co., Ltd. (Shanghai, China). The two cell lines were cultured in RPMI-1640 medium (Hyclone, USA) that was supplemented with 10 % fetal bovine serum (Biological Industries, Israel), and 1 % penicillin and streptomycin (Gibco, USA), respectively. All cells stored at 37 °C in a humidified atmosphere with 5 % CO_2.

2.3. Cell viability

The effect of Huaier on Jurkat and MOLT-4 cell proliferation was measured by CCK8 assay (Dojindo, Japan). Logarithmic growthing cells were inoculated into 96-well plates at 2×10^4/well. Cells were treated with Huaier extract at different concentrations (0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/mL), and cultured for 24, 48 and 72 h. Then, 10 μL of CCK-8 was added to each well for 4 h, and the optical density (OD) value was measured by spectrophotometer (Beckman, USA). Cell viability was calculated as in our previous study [15].

2.4. Cell apoptosis analysis

Cell apoptosis was analyzed by Annexin V FITC Apoptosis Detection Kit I (BD Pharmingen™, USA). Cells were inoculated into 6-well plates at 5×10^5/well, and treated with Huaier for 48 h. The cells were collected, and then washed twice with cold PBS, and resuspended in 1 × binding buffer. Then, FITC Annexin V and PI staining solutions were added and incubated for 15 min. Finally, apoptotic cells were analyzed by flow cytometry (BD Biosciences, USA).

2.5. Hoechst 33258 staining

Jurkat and MOLT-4 cells were co-cultured with Huaier for 48 h, and cells were harvested and washed with PBS. After staining with Hoechst 33258 working solution (Solarbio, Shanghai, China) for 15 min, the nuclear morphology was analyzed by fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Western blot

T-ALL cells were treated with Huaier for 48 h. Cells were harvested by centrifugation, and the supernatant was collected after full

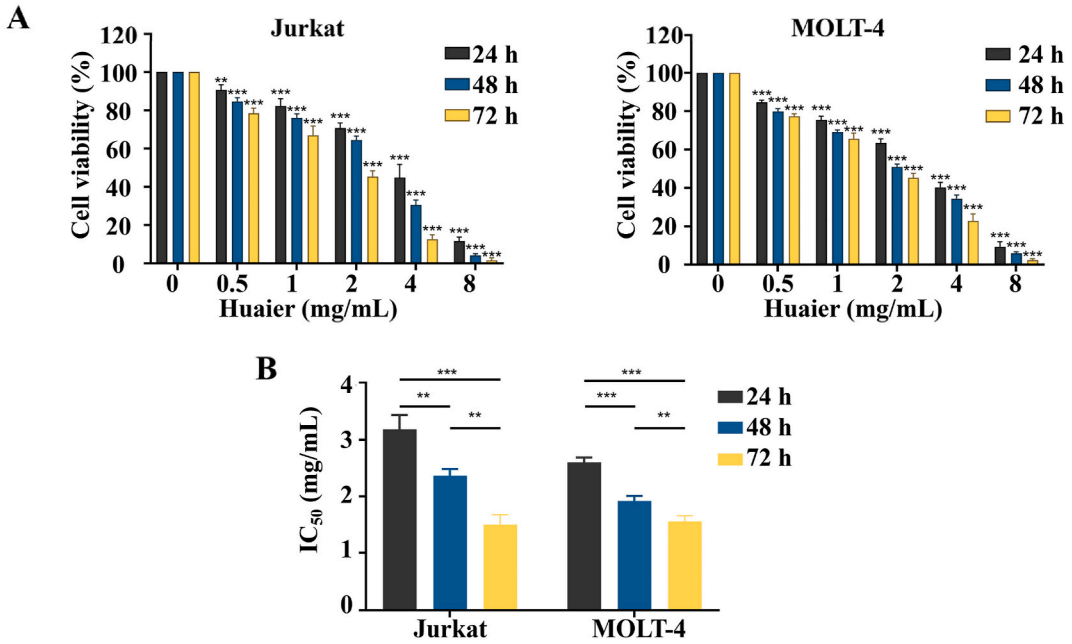

Fig. 1. Huaier inhibits the proliferation of T-ALL cells. (**A**) Cell viability of Jurkat and MOLT-4 cells treated with Huaier for 24, 48 and 72 h. (**B**) The IC_{50} values of Huaier on T-ALL cells. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group.

lysis with RIPA lysate (Beyotime, Shanghai, China) containing protease inhibitors (Roche, Mannheim, Germany). Protein concentrations were measured by BCA method (BioTeke, Beijing, China). Then, 40 μg of protein samples per lane were separated by SDS-PAGE (Solarbio, Beijing, China) and transferred to PVDF membranes (Millipore, USA). Subsequently, the membranes were incubated with antibodies to the target protein overnight at 4 °C. The membranes were washed three times with PBST and incubated with appropriate secondary antibodies (Beyotime, Shanghai, China) for 1 h. Finally, the protein bands were visualized by ECL chemiluminescence system (Vilber, France) [15].

2.7. Statistical analysis

SPSS software (version 20.0) and GraphPad Prism (version 9.5) were performed for statistical analysis. All experiments were repeated three times independently, and data are presented as mean ± standard deviation (SD). LSD-t test and one-way analysis of variance were used to analyze the statistical differences among different groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Huaier inhibits the proliferation of T-ALL cells

To evaluate the effect of Huaier on the proliferation of Jurkat and MOLT-4 cells, cell viability was measured by CCK-8 assay. As shown in Fig. 1A and B, Huaier inhibited the proliferation of Jurkat cells in a dose- and time-dependent manner, and the IC_{50} at 24, 48 and 72 h were 3.18 ± 0.21, 2.37 ± 0.10 and 1.51 ± 0.14 mg/mL, respectively. Huaier suppressed the viability of MOLT-4 cells in a dose- and time-dependent manner, and the IC_{50} at 24, 48 and 72 h were 2.60 ± 0.07, 1.93 ± 0.07 and 1.56 ± 0.08 mg/mL (Fig. 1A and B). These results indicate that Huaier has anti-proliferation effect on T-ALL cells.

3.2. Huaier induces apoptosis in T-ALL cells

First, Jurkat and MOLT-4 cells were stained with Hoechst 33258 staining reagent, and then the morphology of apoptotic cells was monitored by fluorescence microscopy. The results displayed that the apoptotic cells were bright blue, chromatin condensation and nuclear fragmentation, and the number of apoptotic cells increased in a drug concentration dependent manner (Fig. 2A). Subsequently, we quantified the cell proportion of apoptotic cells by flow cytometry, and the results showed that the percentage of apoptotic cells in both cell lines increased significantly after 48 h of Huaier treatment (Fig. 2B). The highest apoptosis rates were 50.67 ± 1.36 % and 49.97 ± 5.43 % in Jurkat and MOLT-4 cells with 4 mg/mL treatment group. The changes in apoptosis rates of Jurkat and MOLT-4 cells were consistent with the morphological results. Furthermore, we analyzed the expression of apoptosis-related proteins, the results showed that the expression levels of Cytochrome *c*, Cleaved caspase-3 and cleaved PARP proteins were significantly increased after Huaier treatment (Fig. 3A). Finally, we performed rescue by using Z-VAD-FMK, a pan-caspase inhibitor, and the results of flow

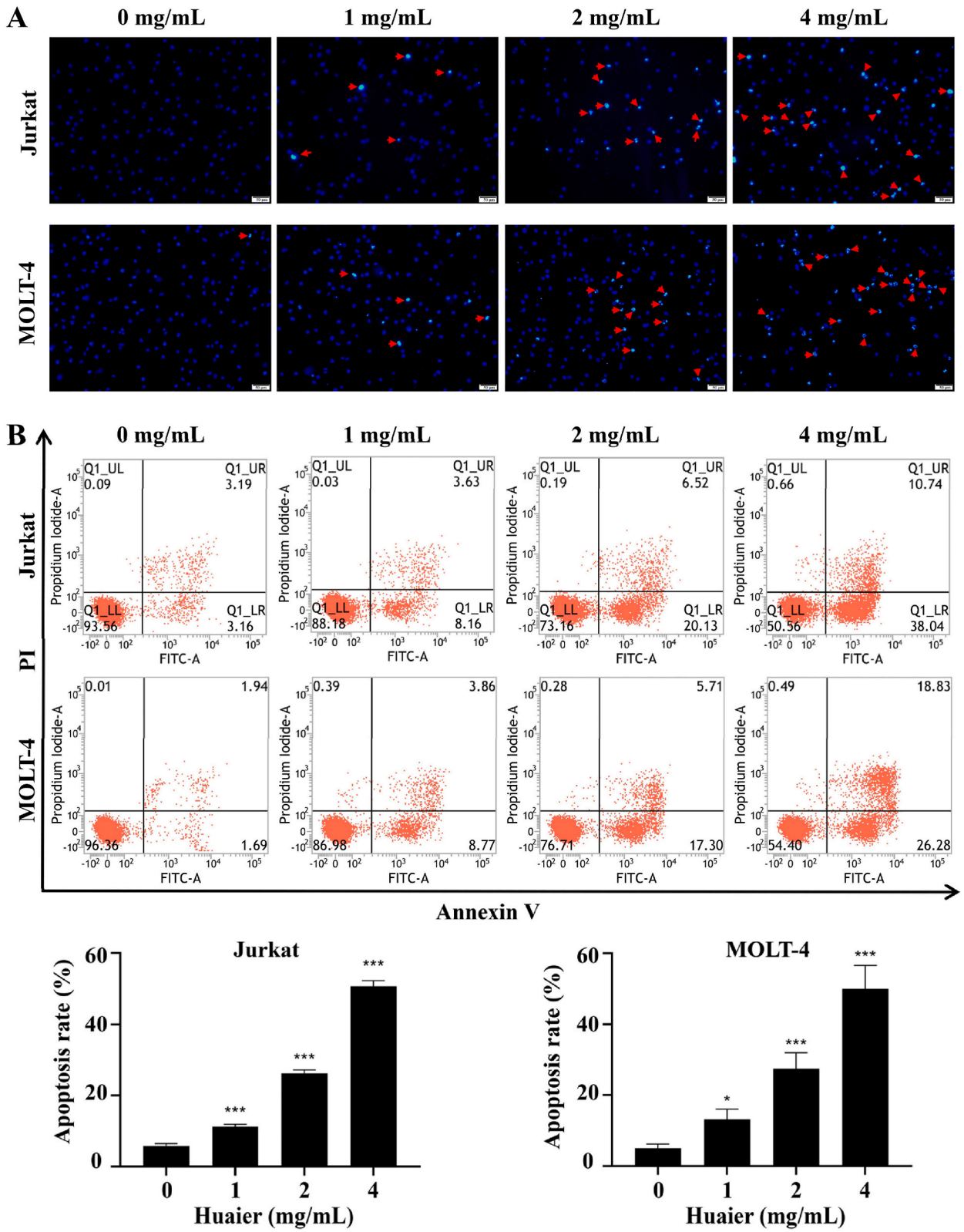

Fig. 2. Huaier induces apoptosis in T-ALL cells. **A** The morphology of apoptotic cells was monitored by fluorescence microscopy (scale bar = 50 μm). **B** The percentage of apoptotic cells was quantified by flow cytometry. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group.

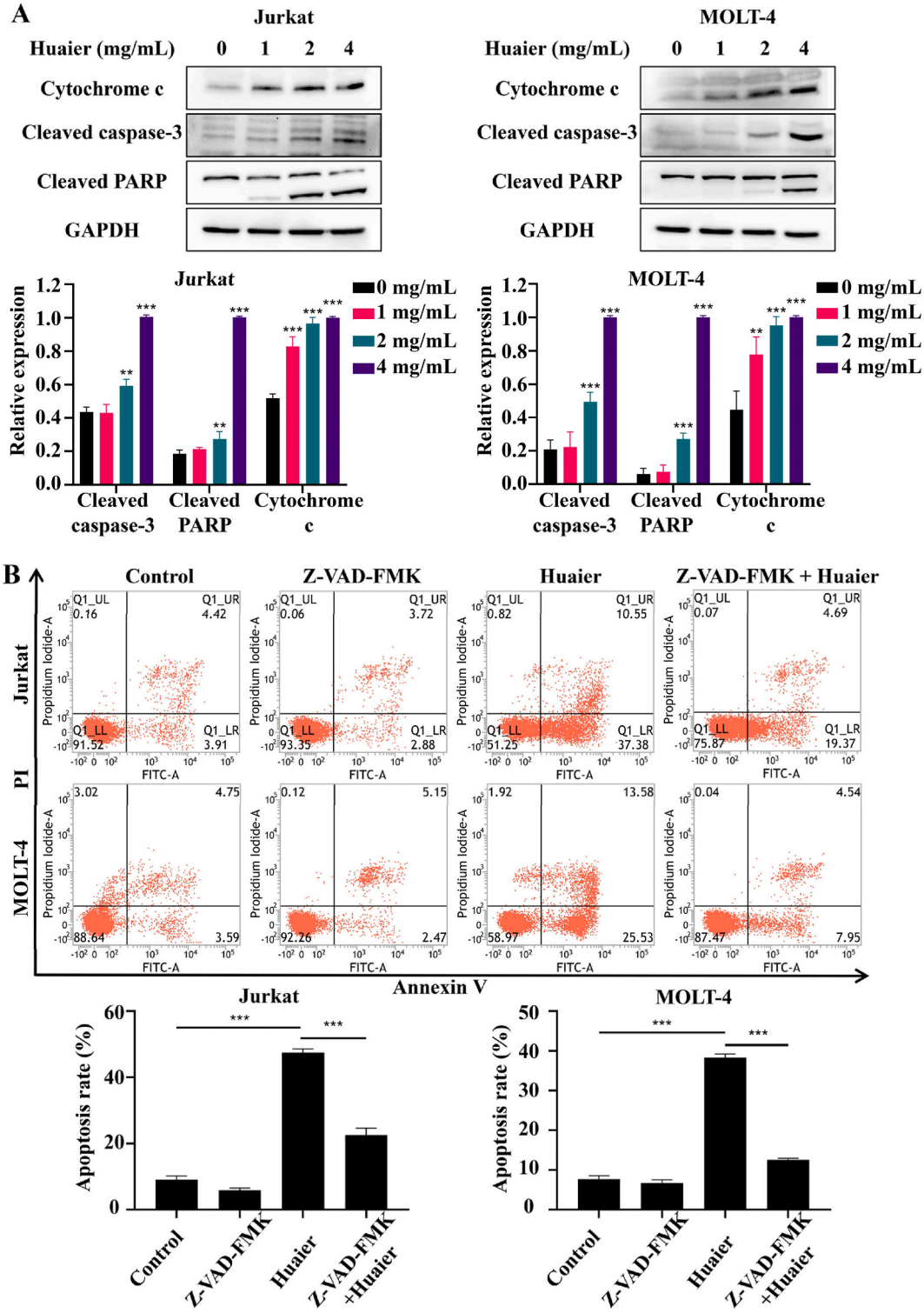

Fig. 3. Huaier induces apoptosis in T-ALL cells. **A** The expression of apoptosis-related proteins in Jurkat and MOLT-4 cells. **B** The apoptosis rates of the two cell lines after Z-VAD-FMK (40 μM) and Huaier (4 mg/mL) treatment. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group.

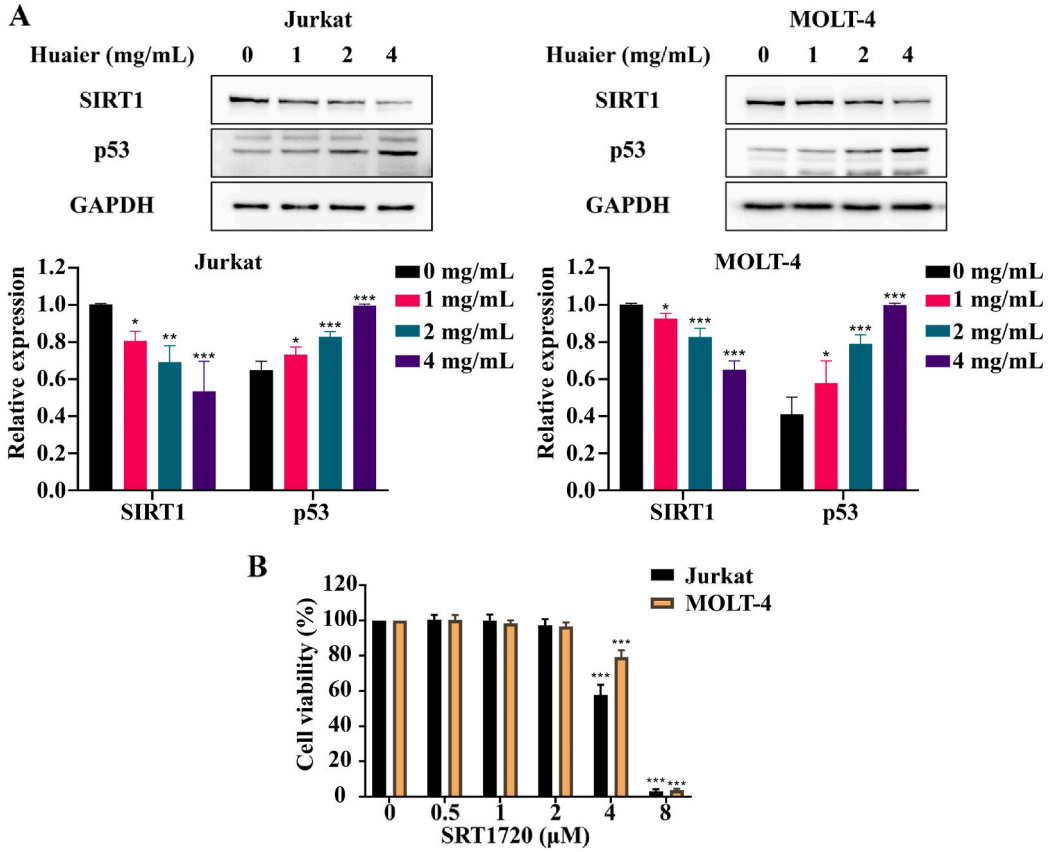

Fig. 4. Huaier inhibits SIRT1 signaling pathway in T-ALL cells. **A** The expression of SIRT1 and p53 proteins after Huaier intervention. **B** Cell viability of Jurkat and MOLT-4 cells treated with SRT1720 for 48 h $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group.

cytometry analysis showed that the proportion of Huaier-induced apoptotic cells was significantly reduced (Fig. 3B). These results indicate that Huaier induces apoptosis in T-ALL cells.

3.3. Huaier induces apoptosis through SIRT1/p53 signaling pathway

To investigate the mechanism of apoptosis induced by Huaier, the expression of SIRT1 and p53 proteins were analyzed by western blotting. The results showed that the expression of SIRT1 protein was decreased, while the expression of p53 protein was increased in both cell lines after Huaier intervention (Fig. 4A). Subsequently, CCK-8 assay results showed that SIRT1 agonist SRT1720 at a concentration of 2 μM or below had no significant inhibitory effect on T-ALL cells (Fig. 4B). Therefore, SRT1720 (2 μM) was used to perform subsequent experiments. Furthermore, the results of flow cytometry showed that the number of Huaier-induced apoptotic cells was significantly reduced after co-treatment of SRT1720 and Huaier (Fig. 5A). Finally, compared with Huaier group, SIRT1 protein expression was increased, p53, Cleaved Caspase-3, and Cleaved PARP protein expression were decreased after treated with SRT1720 and Huaier (Fig. 5B). These results suggest that Huaier induces apoptosis in T-ALL cells by regulating the SIRT1/p53 signaling pathway.

3.4. Huaier inhibits autophagy in T-ALL cells through SIRT1 signaling pathway

We further examined the expression of autophagy-related proteins in the two cell lines after Huaier treatment. The results showed that the expressions of ATG7 and Beclin 1 proteins were decreased, while the expressions of p62 and LC3-II proteins were increased in both cell lines after Huaier intervention (Fig. 6A). After combined treatment with SIRT1 agonist and Huaier, the expressions of LC3-II and Beclin 1 protein were increased, while the expression of p62 protein was decreased in both cells (Fig. 6B). These results indicate that Huaier inhibits autophagy in T-ALL cells.

4. Discussion

Huaier (Trametes robiniophila Murr) is a traditional Chinese medicine with a history of more than 1000 years of clinical application

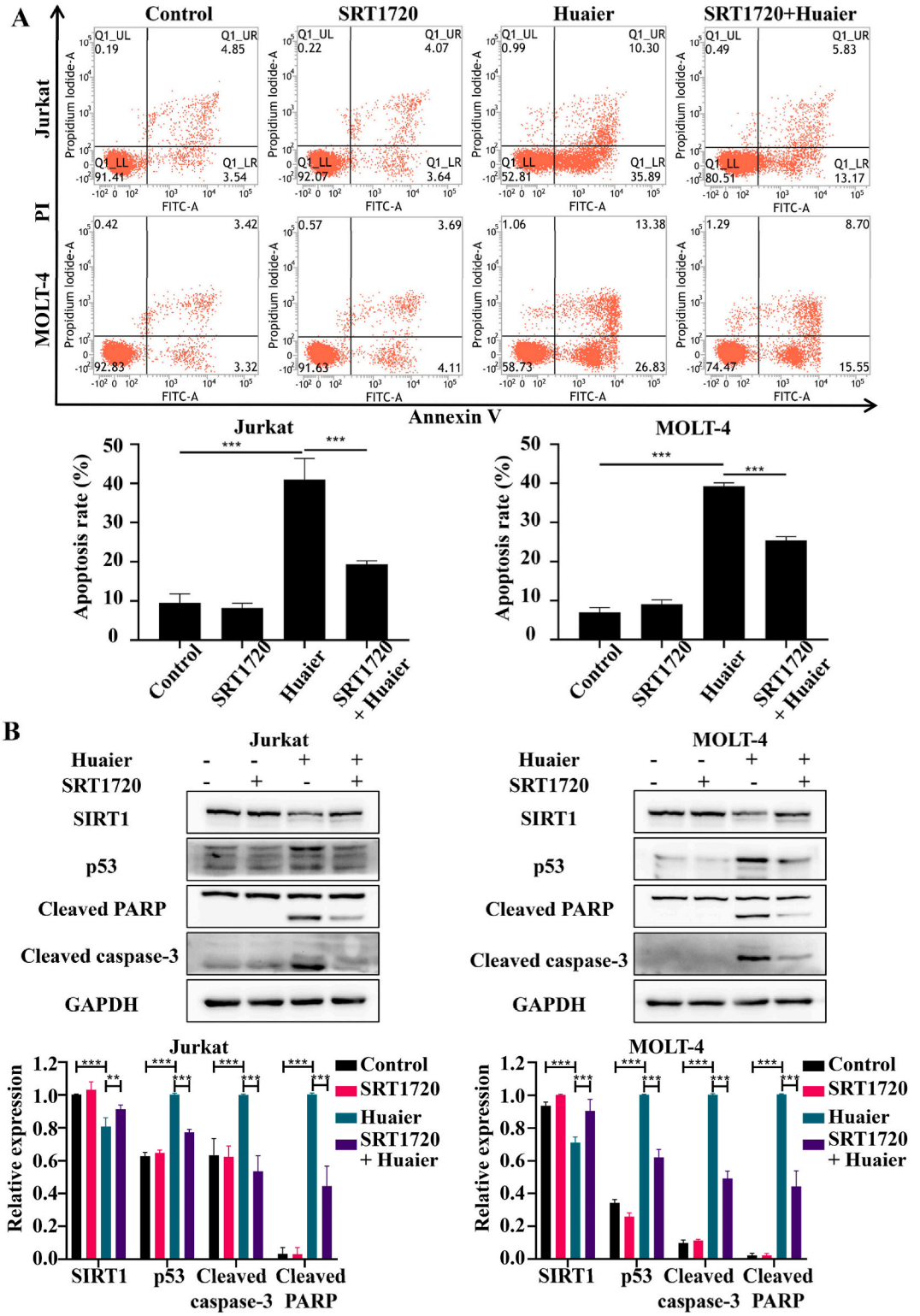

Fig. 5. Huaier induces apoptosis of T-ALL cells through SIRT1/p53 signaling pathway. **A** The apoptosis rate of T-ALL cells treated with SRT1720 (2 μM) and Huaier (4 mg/mL). **B** The expression of related proteins in T-ALL cells after intervention with SRT1720 and Huaier. $^{**}P < 0.01$, $^{***}P < 0.001$.

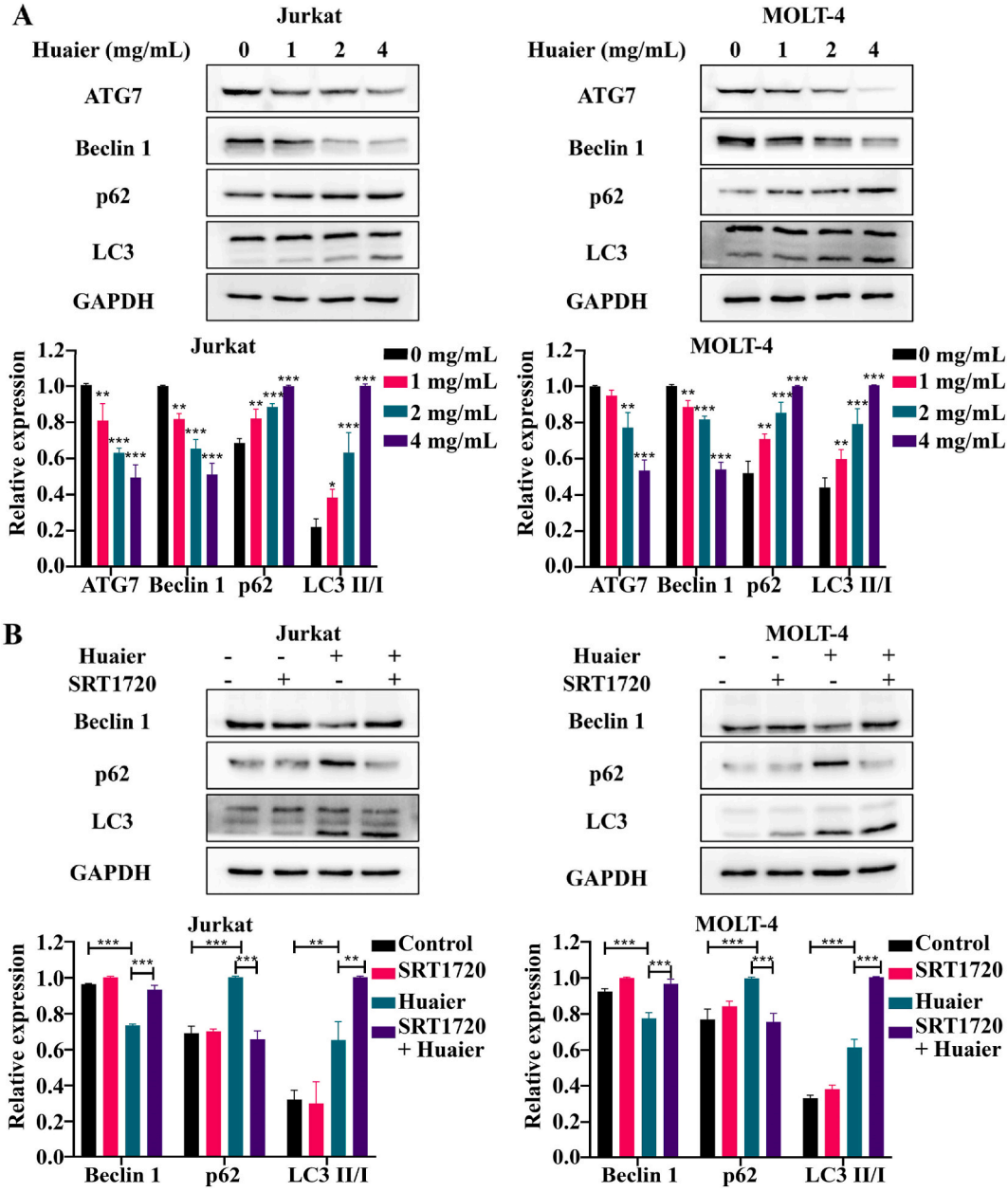

Fig. 6. Huaier inhibits autophagy in T-ALL cells. **A** The expression of autophagy-related proteins in Jurkat and MOLT-4 cell after Huaier treatment. **B** The expression of related proteins in T-ALL cells after intervention with SRT1720 (2 μM) and Huaier (4 mg/mL). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group.

[16]. In recent years, more and more cases of Huaier in the clinical adjuvant treatment of malignant tumors, including hepatocellular carcinoma [9,17], breast cancer [12] and gastric cancer [11]. Numerous clinical studies have confirmed the efficacy of Huaier in improving survival, preventing recurrence, and reducing adverse effects [5]. However, there are few reports on the research of Huaier in hematological tumors. In this study, we investigated the anti-tumor effect of Huaier on T-ALL and its underlying mechanism in vitro.

In the present study, we investigated the anti-proliferative effect of Huaier on T-ALL cells. The results showed that Huaier inhibited the viability of Jurkat and MOLT-4 cells in a dose-dependent and time-dependent manner, with IC_{50} of 2.37 ± 0.10 and 1.93 ± 0.07 mg/mL at 48 h, respectively. Qu, P et al. showed that Huaier also had a similar anti-proliferative effect in B-cell acute lymphoblastic leukemia, with IC_{50} of 3.65 and 5.168 mg/mL for 48 h in Nalm-6 and Sup-B15 cells, respectively [18,19]. These results indicate that Huaier has a significant anti-tumor effect on ALL.

A large number of studies have shown that the anti-tumor mechanisms of Huaier include inhibiting tumor cell proliferation and metastasis, arresting cell cycle, inducing cell apoptosis, pyroptosis, autophagy, anti-tumor angiogenesis, weakening the characteristics

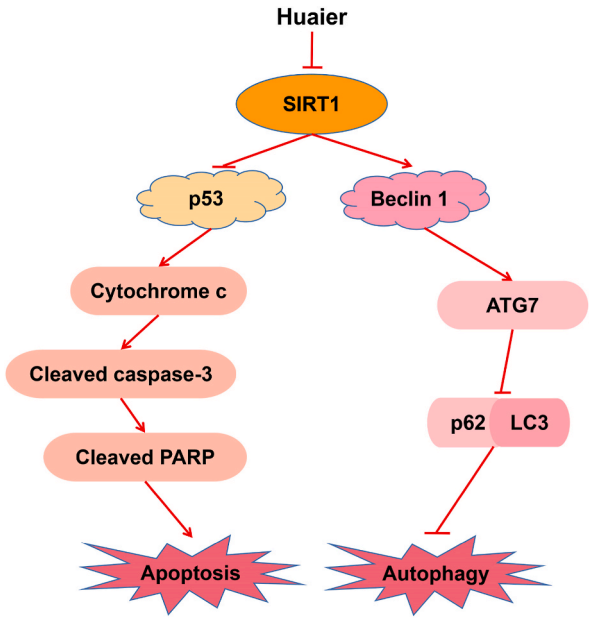

Fig. 7. The mechanism of Huaier against T-ALL.

of tumor stem cell-like cells, and regulating the function of tumor-related immune system [20]. In this study, we evaluated the effect of Huaier on apoptosis in T-ALL cells. Hoechst 33258 staining showed typical morphological changes of apoptotic cells, and quantitative analysis by flow cytometry showed a significant increase in apoptosis rate after Huaier intervention. Mitochondria are the regulatory centers of apoptosis, and various factors such as oxidative stress, DNA damage, calcium regulation imbalance and endoplasmic reticulum stress can initiate mitochondrial pathway mediated apoptosis [21]. Cytochrome *c* is released after mitochondrial damage, which in turn triggers apoptosis mediated by Caspase cascade [22]. Therefore, we further evaluated the expression of apoptosis-related proteins, and the results showed that the expression of Cytochrome *c*, Cleaved caspase-3 and Cleaved PARP proteins were significantly increased after Huaier treatment. In addition, the application of caspase pan-inhibitors rescued Huaier-induced apoptosis. These data suggest that Huaier induces apoptosis in T-ALL.

The tumor suppressor p53 plays an important role in preventing tumorigenesis and inhibiting cancer progression, and it is also an important regulator in the process of cell death [23]. p53 mediates apoptosis through both transcription-dependent and transcription-independent mechanisms [24]. Silent information regulator 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD^+) -dependent deacetylase that plays a crucial role in nuclear events such as transcription, DNA replication and repair [25]. p53 is the first non-histone substrate of SIRT1, and SIRT1 induces the deacetylation of p53 C-terminal residues in a NAD + dependent manner, thereby inhibiting p53-mediated transcription-dependent apoptosis [25]. Studies have shown that SIRT1/p53 signaling pathway is involved in the occurrence and development of a variety of cancers, including leukemia [26],breast cancer [27]. In chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL) and ALL, SIRT1 shows high expression and subsequently regulates tumor fusion proteins leading to carcinogenesis [28]. Moreover, Targeting SIRT1 showed an anti-T-ALL effect [4,29]. Therefore, we investigated the role of SIRT1/p53 signaling in Huaier anti-leukemia. The results showed that Huaier inhibited SIRT1 protein expression and increased p53 protein expression, and SRT1720 (SIRT1 agonist) rescued Huaier-induced apoptosis. These results indicate that Huaier can induce apoptosis in T-ALL cells by inhibiting SIRT1/p53 signaling pathway.

SIRT1 has long been known to play a crucial role in regulating autophagy through its deacetylase activity. SIRT1 mediates autophagy not only by interacting with upstream regulators of autophagy initiation, elongation, maturation, fusion and degradation processes [30], such as SIRT1 inducing autophagic cell death by regulating phosphatidylinositol 3-kinase catalytic subunit 3/VPS34-Beclin 1 complex [31]. Moreover, SIRT1 can directly deacetylate ATGs (ATG3, ATG5, ATG7) and LC3 to induce autophagy [28,30]. In this study, the expression of Beclin 1 and ATG7 was inhibited, and the expression of p62 and LC3-II was increased by Huaier, which could be reversed by SIRT1 agonist SRT1720. It has been reported that the expression of p62 and LC3-II is simultaneously up-regulated, indicating that autophagy flow is blocked [32,33]. Therefore, our results showed that Huaier inhibited the occurrence of autophagy by down-regulating SIRT1 expression.

5. Conclusion

Taken together, Huaier promotes apoptosis and inhibits autophagy in T-ALL cells by inhibiting SIRT1 signaling pathway (Fig. 7). This study suggests that Huaier may be a potential drug for the treatment of T-ALL, and SIRT1 may be a potential target, but the anti-leukemia effect of Huaier in vivo and its clinical efficacy still need to be further investigated.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Declaration of interest's statement

The authors declare no competing interests.

CRediT authorship contribution statement

Xiang Qin: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Xi Chen:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Fan Wang:** Writing – review & editing, Software, Formal analysis, Data curation, Conceptualization. **Fangfang Zhong:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Yan Zeng:** Writing – review & editing, Project administration, Conceptualization. **Wenjun Liu:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations:

T-ALL	T-cell acute lymphoblastic leukemia
CML	chronic myeloid leukemia
CLL	chronic lymphocytic leukemia
SIRT1	silent information regulator 1
PARP	poly ADP-ribose polymerase
NAD	nicotinamide adenine dinucleotide;
ATG	autophagy related protein

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37313.

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