# Rhein regulates the proliferation and apoptosis of human leukaemia cells and its effects on the miR-27/CUL5 axis

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## Abstract

**Introduction:** This study was undertaken to examine the anticancer effects of the natural product rhein on human leukaemia cells.
**Material and methods:** Cell viability was determined by MTT assay. Immunofluorescence staining, DAPI, AO/EB, and annexin V/PI staining were used for the detection of apoptosis. qRT-PCR analysis was used to examine protein expression. Western blot analysis was used to determine protein expression.
**Results:** The results revealed that rhein caused a significant ($p < 0.05$) and dose-dependent decrease in the viability of AML-193 leukaemia cells. It was also revealed that rhein caused morphological changes such as cell rounding in rhein-treated AML-193 cells. The DAPI and AO/EB staining showed that rhein caused remarkable changes in the nuclear morphology of the AML-193 cells, characteristic of apoptosis. The percentage of apoptosis was found to be 3.12%, 14.80%, 30.31%, and 60.85% at the concentrations of 0, 3.5, 7, and 14 µM of rhein, respectively. The expression of Bax, cleaved caspase-3, 9, and cleaved PARP increased concentration dependently, while the expression of Bcl-2 decreased. The effects of rhein were also examined on 11 different miRs, and it was found that rhein specifically and significantly ($p < 0.05$) inhibited the expression of miR-27. Additionally, inhibition of miR-27 resulted in the decrease of AML-193 viability and upregulation of Cullin 5 (CUL5).
**Conclusions:** Taken together, rhein triggers apoptosis in leukaemia cells and modulates the miR-27/CUL5 axis. As such, rhein may prove to be beneficial in the treatment of leukaemia.

**Key words:** leukaemia, apoptosis, proliferation, rhein, expression, microRNA.

## Introduction

Plants are nature's best chemists, with the capacity to produce an amazing diversity of molecules. These molecules help the plant to defend itself from the challenging biotic and abiotic stress [1]. Over the years, human beings have utilised these natural compounds for their own benefit, especially in healthcare [2]. Many antimicrobial, anticancer, and antimalarial agents are natural plant products [3]. Rhein is an important plant secondary metabolite with exceptional pharmacological potential [4]. Several studies have reported the anticancer effects of rhein. Duraipandiyan *et al.* reported the anticancer effects of rhein isolated from the flowers of Cassia

fistula [5]. Fonteneau *et al.* also reported the anticancer effects of rhein analogues [6]. Wang *et al.* reported that rhein induces apoptosis in cancer cells via FOX-mediated enhancement of Bim expression [7]. In yet another study, rhein was shown to target the STAT3 signalling cascade to suppress the growth of the non-small cell lung cancer cells [8]. Nonetheless, the anticancer effects of rhein have not been reported against human leukaemia cells. Consistently this study was designed to investigate the anticancer effects of rhein against human leukaemia cells and to decipher the molecular mechanism underlying the anticancer effects of rhein. Leukaemias are the most prevalent childhood malignancies. They account for approximately 30% of all the paediatric malignancies with acute lymphoblastic leukaemias constituting about 76% of all cases of acute leukaemia [9]. In many developing countries, such as Nigeria, leukaemia accounts for about 10.2% to 12.9% of childhood malignancies [10]. Additionally, the majority of currently used chemotherapeutic agents have side effects [11].

In this study we, for the first time, show that rhein inhibits the growth of human leukaemia cells via inhibition of cell proliferation and promotion of cell apoptosis. These effects of rhein were found to be mediated via modulation of the miR-27/CUL5 axis. Taken together, rhein may prove beneficial in the treatment of leukaemia and warrants further research studies.

## Material and methods

### Cell lines and culture conditions

Leukaemia AML-193 and normal NCI-H526 normal cell lines were procured from ATCC, USA. The culturing of cell lines was performed using Dulbecco's modified Eagle medium (DMEM, Thermo Scientific). The cell lines were maintained in a $CO_2$ incubator at 37°C with 5% $CO_2$ concentration and relative humidity of 98%.

### Cell proliferation estimation assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Thermo Fisher Scientific) was used for determination of proliferation of normal and leukaemia treated with 0 to µM rhein for 24 h in 96-well plates with $1 \times 10^6$ cells/well. 15 µl of MTT solution was added to each well. After 2 h incubation at 37°C, absorbance values were recorded for each sample at 50 nm with a spectrophotometer.

### Immunofluorescence for cleaved aspase-3 analysis

The rhein-treated AML-193 cells were cultured and subjected to fixation on coverslips with paraformaldehyde for 20 min at 25°C. This was followed by washing with PBS incubation for 30 min with 1% bovine serum albumin (BSA) to prevent the blocking of antibodies non-specifically. The cells were then subjected to incubation with primary antibody (1 : 200 dilution) and washed with PBS. Next the cell samples were subjected to incubation with Alexa Fluor 488-conjugated goat antirabbit IgG secondary antibody (1 : 200) in 1% BSA for 1 h in the dark at 25°C. The samples were then subjected to counterstaining with DAPI for 7 min. Finally, the coverslips were subjected to visualisation using a fluorescence microscope (200× magnification).

### Annexin V/PI staining method

The 100 µl of AML-193 cell culture containing about $1 \times 10^6$ cells was added to each of the wells of the 96-well plate. Then, to each well 0, 3.5, 7, or 14 µM rhein was added and 37°C incubation was applied for 24 h. The samples were then centrifuged, and cell pellets were suspended in PBS buffer after being washed with PBS and fixed with 4% paraformaldehyde. 30 µl of the cell suspension was transferred to a sterile glass slide, and the cells were stained with annexin V/PI (Sigma-Aldrich). The cells were examined under a fluorescent microscope for the detection of apoptosis levels.

### RT-PCR-based expression analysis

Total RNA was isolated using TRIzol (Invitrogen) reagent. This was followed by DNAse I treatment and cDNA synthesis using a High-Capacity cDNA Archive™ Kit following the manufacturer's protocol. With human GAPDH gene as the internal control of gene expression, RT-PCR-based expression analysis was performed using SYBR Green method. The amplification conditions were 10 min initial denaturation at 95°C followed by 40 cycles of 30 s denaturation at 95°C, 30 s of primer annealing at 58°C, and 20 s of extension at 72°C.

### Transfection of cancer cells

RiboBio (Guangzhou, China) was used for synthesis of miR-NC and miR-16 mimics. Transfection of AML-193 cancer cells was performed using Lipofectamine 2000 (Invitrogen) reagent following the manufacturer's protocol. When 80% of cell confluence was attained post transfection, the cells were PBS washed followed by treatment with 0.25% trypsin to obtain a homogenous cell suspension.

### Western blotting

Total proteins were isolated from leukaemia cells treated with 0, 3.5, 7, or 14 µM rhein for 24 h.

The total cellular proteins were extracted with RIPA lysis and extraction buffer (Thermo Fisher Scientific) and then quantified by using Bradford's reagent. Using the electrophoretic method, 40 µg of proteins were separated on 12% SDS-PAGE gels. The gel contents were then transferred to the PVDF membranes via blotting method. The membranes were exposed to primary antibodies followed by exposure of secondary antibodies at 4°C in the dark. With human actin gene as normalization control, the protein band detection and estimation of relative concentrations was made by employing the use of an efficient chemiluminescent reagent.

### Statistical analysis

The mean and standard deviation (SD) values were calculated from the data obtained from replicas of experimental setups, and final representation was made as mean ± SD. GraphPad Prism 7 software was used to perform *t* tests. A *p*-value less than or equal to 0.05 was taken as an indicator of statistically significant difference.

## Results

### Rhein inhibits the proliferation of human leukaemia cells

The proliferation of AML-193 leukaemia cells was assessed at different concentrations of rhein (Figure 1 A) by the MTT assay. Rhein caused a remarkable decline in the viability of the AML-193 cells. The growth inhibitory effects of rhein showed a concentration-dependent pattern (Figure 1 B). However, the effects of rhein on the normal NCI-H526 cells were less profound. The $IC_{50}$ of rhein against the human leukaemia cells was found to be 7 µM, which is indicative of strong anti-proliferative effects of rhein on the leukaemia cells (Figure 1 B). Additionally, morphological analysis of the rhein-treated AML-193 cells showed that rhein caused significant changes in the morphology of the AML-193 cells, which included membrane blebbing and rounding of the cells (Figure 2).

### Rhein promotes apoptosis of the human leukaemia cells

Because rhein caused a remarkable decline in the proliferation rate of the human leukaemia cells, we wanted to gain insights about the molecular mechanism underlying the anticancer effects of rhein against human leukaemia cells. Therefore, we performed different staining assays. The results of immunofluorescence imaging and counterstaining with DAPI staining showed that rhein caused remarkable changes in the nuclear morphology of the AML-193 cells and increased the expression of cleaved caspase-3 indicative of apoptosis (Figure 3). The AO/EB staining showed that rhein caused an increase in the orange colour cells, indicating that rhein induces apoptosis in the AML-193 cells (Figure 4). Next, to assess the extent of apoptosis, annexin V/PI staining assay showed that the apoptotic cells increased in a dose-dependent pattern. The percentages of apoptosis were found to be 3.12%, 14.80%, 30.31%, and 60.85% at the concentrations of 0, 3.5, 7, and 14 µM of rhein (Figure 5). Western blotting analysis of the rhein-treated AML-193 cells showed that the expression of Bax, cleaved caspase-3, cleaved caspase-9, and cleaved PARP increased considerably and in a concentration-dependent manner, and the expression of Bcl-2 decreased concentration dependently (Figure 6).

### Rhein modulates the miR-27/CUL5 axis

The effects of rhein were also examined on the expression of 11 different microRNAs (miRs). The

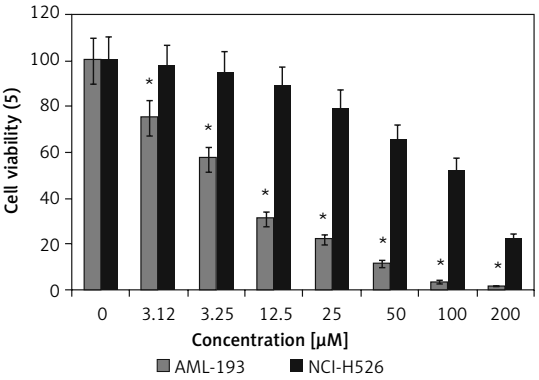


**Figure 1.** MTT assay showing the effects of Rhein on leukaemia AML-193 and normal NCI-H526 cells. The experiments were performed in triplicate and expressed as mean ± SD (*$p < 0.05$)

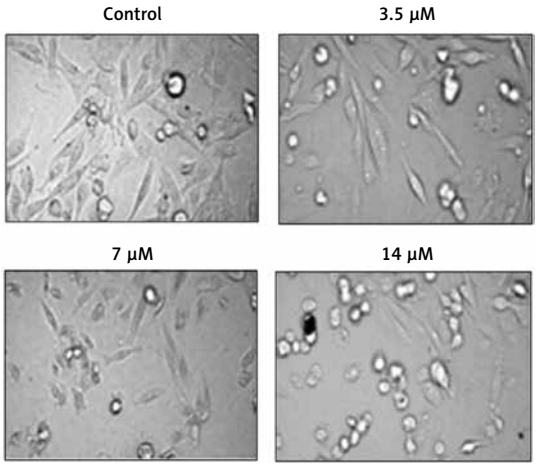


**Figure 2.** Effects of Rhein the morphology of leukaemia AML-193 cells at indicated doses. The experiments were performed in triplicate

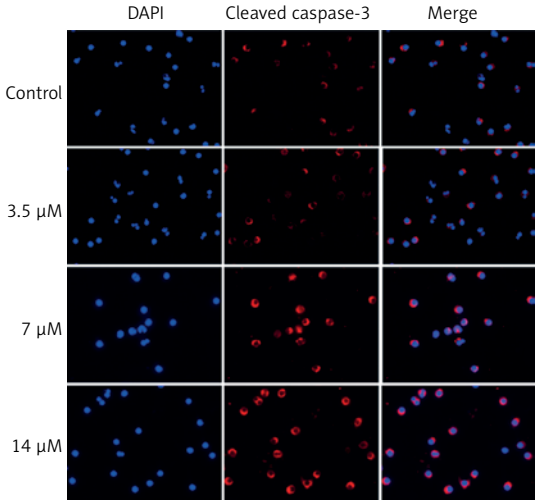


**Figure 3.** Immunofluorescent analysis of cleaved caspase-3 and DAPI staining showing that rhein induces apoptosis by increasing cleaved caspase-3 expression in AML-193 cells at indicated doses. The experiments were performed in triplicate

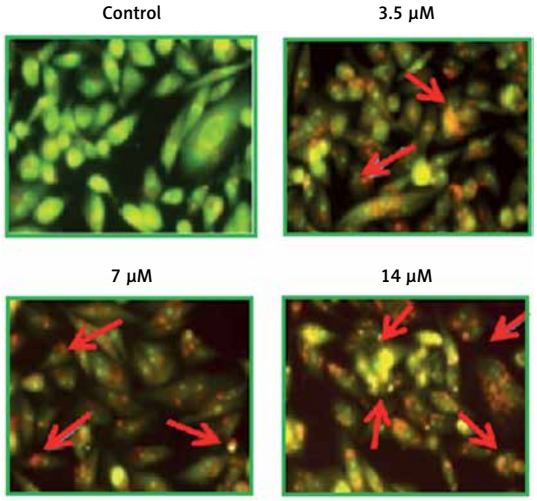


**Figure 4.** AO/EB staining showing the rhein induces apoptosis in AML-193 cells at indicated doses. The experiments were performed in triplicate

results showed that rhein specifically caused the downregulation of miR-27 in the AML-193 cells (Figure 7 A). Therefore, we speculated that rhein exerted its effects on the proliferation of AML-193 cells by suppressing miR-27. To confirm this we silenced the expression of miR-27 in AML-193 cells, and interestingly we found that silencing of miR-27 also caused inhibition of the AML-193 cell proliferation similar to that of rhein treatment (Figure 7 B). TargetScan analysis showed that miR-27 targets Cullin 5 (CUL5) in AML-193 cells (Figure 7 C), and western blot analysis also showed upregulation of CUL5 upon miR-27 inhibition (Figure 7 D).

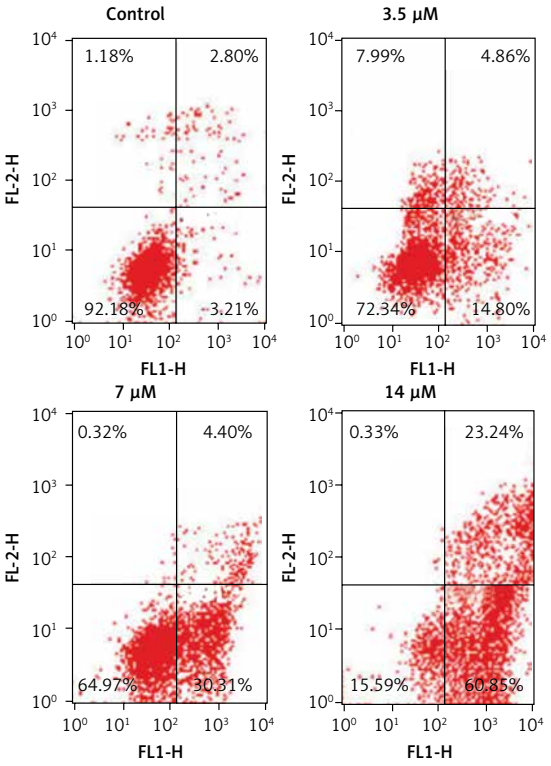


**Figure 5.** Annexin V/PI staining showing that rhein induces apoptosis in AML-193 cells at indicated doses. The experiments were performed in triplicate

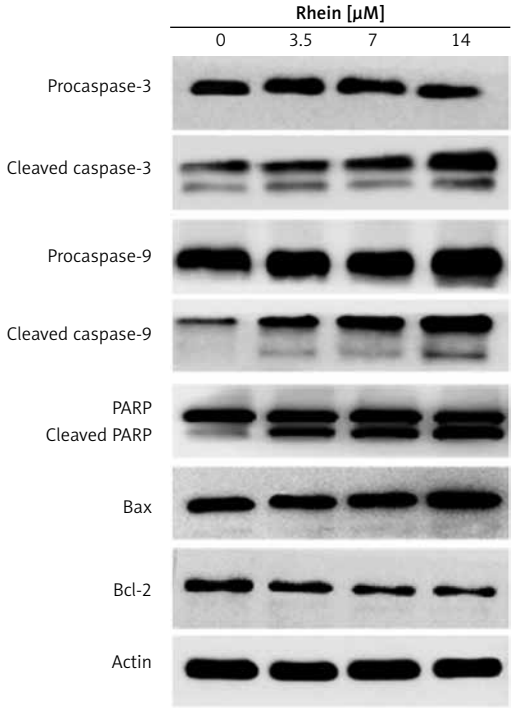


**Figure 6.** Western blots showing the expression of apoptosis-related proteins in leukaemia AL-193 cells. The experiments were performed in triplicate

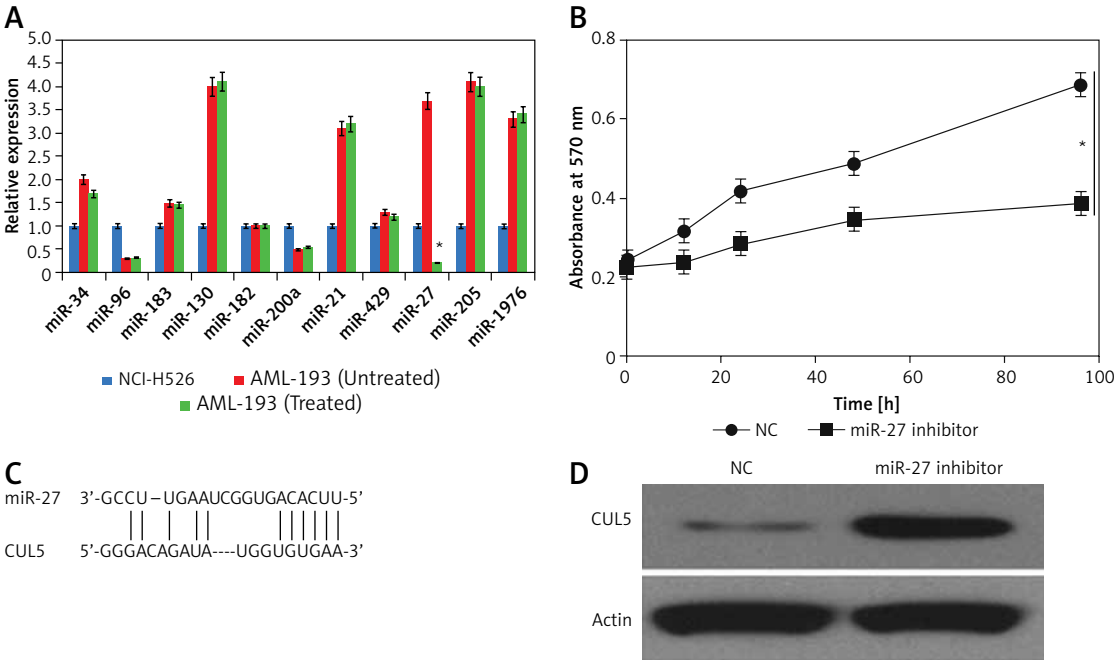


**Figure 7. A** – Expression of different microRNA in normal NCI-H526, rhein-treated and untreated AML-193 cells. **B** – Cell viability of NC and miR-27 inhibitor transfected AML-193 cells. **C** – TargetScan analysis showing CUL5 as the target of miR-27. **D** – Western blot showing the expression of CUL5 in NC and miR-27 inhibitor transfected AML-193 cells. The experiments were performed in triplicate and expressed as mean ± SD (*$p < 0.05$)

## Discussion

Leukaemia constitutes approximately 3% of all cancer cases globally [12]. The incidence of leukaemia has been reported as comparatively higher in children and adults [12]. The chemotherapeutic agents currently used for the treatment are inefficient and exhibit numerous side effects [13]. Therefore, there is a continuous pursuit to look for novel and efficient natural compounds that could be utilised for the treatment of leukaemia. Rhein is an important plant-derived compound with enormous pharmacological potential [4]. Numerous studies have reported the anticancer effects of rhein on different types of cancer cells [7]. Nonetheless, the anticancer effects of rhein have not been thoroughly explored against human leukaemia cells. This study was designed to investigate the effects of rhein on the proliferation of the human leukaemia cells. Interestingly, it was found that rhein caused a remarkable decrease in the viability of the AML-193 cells. However, the effects of rhein on the normal NCI-H526 cells were significantly low. These observations are in agreement with several studies carried out earlier. For instance, it was reported that rhein causes inhibition of the growth of non-small cell lung carcinoma [8]. The exploration of the molecular mechanisms responsible for the anticancer effects of rhein were also explored, and was it found that rhein induces apoptosis in the AML-193 leukaemia cells. Apoptosis is an important mechanism to eliminate the defective cells from the body of the organism, and there are several marker proteins for the onset of apoptosis [14]. Herein we examined the effect of rhein on the expression several marker proteins, and we found that the expression of Bax, cleaved caspae-3 and -9, and cleaved PARP was remarkably enhanced, while that of Bcl-2 was suppressed, further confirming that rhein induced apoptosis in the leukaemia AML-193 cells. MicroRNAs (miRs) have been reported to exhibit enormous therapeutic potential for the management of human diseases and disorders because of their diverse regulatory roles [15]. Recently miRs have gained importance due to their potential in cancer treatment [16]. Herein, we examined the effects of rhein on the expression of 11 different miRs. It was found that rhein specifically inhibits the expression of miR-27. Additionally, inhibition of miR-27 resulted in the decrease of AML-193 viability and also upregulated the expression of CUL5. These findings are supported by previous studies wherein miR-27a has been shown to be targeted by anticancer agents to inhibit the growth of colon cancer cells [17]. Taken together, rhein may prove beneficial in the treatment of leukaemia.

In conclusion, the present study revealed that rhein significantly and specifically inhibits the growth of the human leukaemia cells via apoptosis induction. Additionally, it was found that rhein exerts its effects by modulating miR-27/CUL5 expression.

## Conflict of interest

The authors declare no conflict of interest.

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