

RESEARCH ARTICLE

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Regulation of Apoptosis by SYB in HepG2 Liver Cancer Cells is Mediated by the P53/Caspase 9 Axis



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Abstract: Objective: To explore the function of miR-34a in promotion of apoptosis by SYB.**Methods:** In this study, the most effective concentration of SYB was determined by measuring cell proliferation. Relative miR-34a mRNA levels were detected by quantitative RT-PCR. Apoptosis was assessed using Annexin-V/PI assays, whereas protein levels of p53, caspase 3, caspase 9, caspase 8 and Bcl2 were evaluated by western blotting.**Results:** Minimum HepG2 cell growth was observed after 36h of exposure to 150 nmol/L SYB. miR-34a expression was highest 40min after the addition of SYB. SYB slightly decreased the abundance of Bcl-2, but increased the abundance of p53, caspase 3, caspase 9 and caspase 8. SYB failed to alter miR-34a expression when p53 was inhibited. Bcl-2 abundance remained low over time, whereas the abundance of caspase 3, caspase 9 and caspase 8 gradually increased. Inhibition of p53 promoted HepG2 cell growth in comparison with that of the control group. miR-34a was silenced to assess the role of miR-34a in the inhibitory effect of SYB on HepG2 cell growth. When p53 was silenced, protein abundance of Bcl2, caspase 3, caspase 8 and caspase 9 remained unchanged following the addition of SYB; moreover, HepG2 cell growth was increased.**Conclusion:** SYB represents a promising therapeutic approach for liver cancer patients.

ARTICLE HISTORY

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10.2174/1871520617666170327161433**Keywords:** SYB, p53, Caspase 9, HepG2, miR-34a.

1. INTRODUCTION

Liver cancer is a fatal malignant disease and constitutes the third leading cause of tumor mortality worldwide [1, 2]. Therefore, liver cancer has become a major health concern. Surgery is the conventional approach to liver cancer treatment [3]. Owing to the high invasiveness of liver cancer, most cases have already progressed to the intermediate and advanced stages by the time of diagnosis, meaning that the optimal time for surgery has passed [4]. The resection rate for liver cancer is less than 15%, while the postoperative recurrence rate is approximately 50% [5, 6].

Recently, microRNAs have been shown to regulate the expression of numerous genes, including tumor suppressor genes and oncogenes such as p53 and Bcl2 [7, 8]. However, despite extensive research, the functional role of microRNAs is not completely understood. Many miRNAs play a role in regulating apoptosis [9]. Recent studies have demonstrated relationships between changes in apoptosis-related gene expression and miRNA expression during cancer therapy; for example, miR-34a and p53 regulate each other [9-11].

A member of the compositae family, *Carthamus tinctorius* L. (safflower) has long been used in Mongolian medicine and Traditional Chinese Medicine [12-14]. Safflower contains lignans, flavonoids, triterpene alcohols, and polysaccharides [15-18]. Safflower has been used in the treatment of cardiovascular diseases

for many years [19, 20]. As one of the main bioactive constituents of safflower, safflower yellow B (SYB) has been used extensively in the treatment of cardiocerebrovascular diseases in Mongolian medicine for many years. However, little research on the effects of SYB on liver preservation has been undertaken. Thus, the aim of the present study was to determine whether SYB promotes cell death in tumoral hepatocytic cells and the function of miR-34a in this effect. Remarkably, here we report that SYB directly regulates transcription of miR-34a. We demonstrate that exposure to SYB promotes apoptosis and leads to dramatic global alterations in miR-34a expression.

2. MATERIALS AND METHODS

SYB (purity >98%) was purchased from the Chinese National Institute (Beijing, China) (Fig. 1). Primary antibodies (p53, caspase 3, caspase 9, caspase 8 and Bcl2) were purchased from Abcam Co. (Cambridge, MA, USA). Secondary antibodies were purchased from Cytomatin Gene Company (Isfahan, Iran). Annexin-V-FLUOS Staining kits were purchased from Roche (Roche, Mannheim, Germany). qRT-PCR master mix was purchased from Takara (Shiga, Japan). miRNA expression assay kits were purchased from Exiqon. p53 inhibitor Pifithrin- α was purchased from Abcam Co. (UK).

3. CELL CULTURE

HepG2 and Hep3B liver cancer cells (Chinese Academy of Sciences, China) were cultured at 37°C in a humidified atmosphere consisting of 5% CO₂. The culture media was DMEM containing 10% fetal bovine serum (Gibco, USA). A p53 inhibitor (150 nM) was used to inhibit p53. The siRNA silencing time was 36 h.

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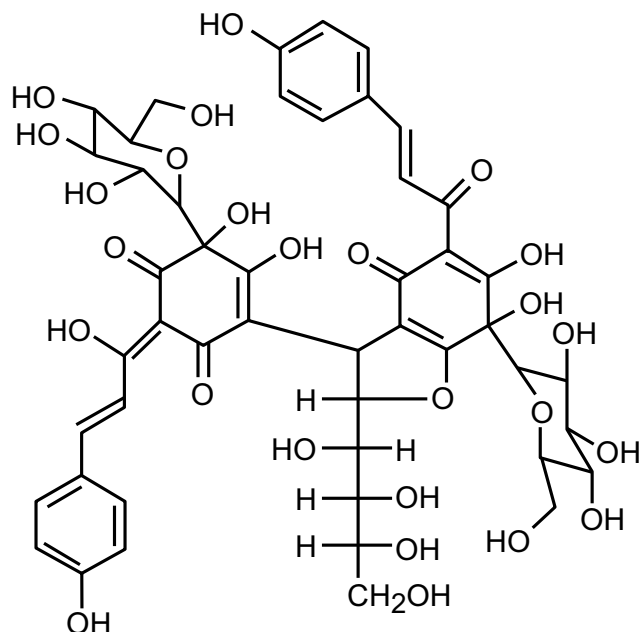


Fig (1). The chemical structure of SYB.

4. CELL PROLIFERATION ASSAY

The Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability and proliferation. Briefly, cells were seeded in 96-well plates (3000 cells/well, four replicates) and incubated with different final concentrations of SYB (0, 50 nmol/L, 100 nmol/L, 150 nmol/L, 200 nmol/L) for different periods of time (12 h, 24 h, 36 h, and 48 h). The cells were replenished with medium containing CCK-8 solution (10 μ L CCK-8 in 100 μ L medium) and incubated for 2 h, after which the absorbance at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The survival rate of the cells was calculated as the ratio of the normalized absorbance to that of the non-irradiated controls.

5. WESTERN BLOTTING

Cellular protein samples were separated *via* 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies overnight at 4°C. Next, the membranes were washed three times with PBS, incubated with peroxidase-conjugated secondary antibodies, and developed using ECL reagents (Pierce, Rockford, IL, USA).

6. FLOW CYTOMETRIC ANALYSIS

Cells (1×10^6) were washed by PBS 48 h after the addition of SYB, after which they were labeled by Annexin V and propidium iodide (PI). Apoptotic rates were measured using flow cytometry and Flowjo software. The percentage of apoptotic cells was measured by applying the criteria of Annexin V-positivity and PI-negativity, while the percentage of late apoptotic cells was determined by applying the criteria of Annexin V-positivity and PI-positivity.

7. QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ANALYSIS (qRT-PCR)

Total cellular RNA was obtained using AccuZol reagent (Bioneer, Daedeok-gu, Daejeon, Korea). Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was per-

formed using a Rotor-Gene 6000 system (Corbett Life Science, Mortlake, NSW, Australia) with SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) [21]. The PCR reaction system consisted of 5 μ L of SYBR green reagent, 0.2 μ M of each primer, 1 μ L of cDNA template, and 6 μ L of nuclease-free water. The mRNA level was calculated by the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = Ct_{\text{sample}} - Ct_{\beta\text{-actin}}$). The primer sequences are listed in Table 1.

8. STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was performed using GraphPad Prism version 5 software (GraphPad Software, La Jolla, CA, USA, www.graphpad.com/company/). Data are presented as mean \pm S.D. P-values less than 0.05 were considered significant.

9. RESULTS

9.1. Effect of SYB on HepG2 Cell Growth and the Optimal SYB Concentration

The effects of 0, 50 nmol/L, 100 nmol/L, 150 nmol/L, and 200 nmol/L (final concentration) SYB on HepG2 cell growth were assessed after 12 h, 24 h, 36 h, and 48 h of exposure. HepG2 cell growth was directly related to the exposure time and concentration of SYB; minimum HepG2 cell growth was observed after 36 h of exposure to 150 nmol/L SYB (Fig. 2A). The growth percentage of Hep3B cells (Hep3B cells do not express p53) exposed to SYB was not different from that of the blank group (Fig. 2B).

To identify the concentration at which SYB most effectively induced HepG2 cell apoptosis, flow cytometric analysis was coupled with Annexin V/PI staining. The most effective concentration of SYB was 150 nmol/L (Fig. 3A and B).

9.2. Effect of SYB on miR-34a

qRT-PCR was performed to evaluate the effect of SYB on miR-34a expression. Exposure to SYB increased miR-34a expression over time (Fig. 4). miR-34a expression peaked 40 min after the addition of SYB.

9.3. SYB Inhibits HepG2 Cell Growth *via* the p53/caspase 9 Axis

To determine the signaling pathway(s) mediating the inhibitory effect of SYB on HepG2 cell growth, protein levels of p53, caspase 3, caspase 9, caspase 8, and Bcl2 were detected after 0, 20, 40, and 60 min of exposure to 150 nmol/L SYB (Fig. 5A). SYB decreased Bcl-2 abundance only slightly, whereas protein levels of p53, caspase 3, caspase 9, and caspase 8 were increased by exposure to SYB (Fig. 5B (a-e)), suggesting that p53/caspase 9 signaling plays a key role in the inhibitory effect of SYB on HepG2 cell growth. To verify this result, a P53 inhibitor was used to silence P53 protein expression in addition to SYB exposure. miR-34a expression was lower in HepG2 cells following exposure to SYB in combination with an inhibitor of p53 expression (Fig. 6A). Bcl-2 expression remained low over time in cells exposed to SYB in combination with an inhibitor of p53 expression (150 nM), but the expression level of Bcl-2 in such cells was higher than that of cells exposed to SYB without the p53 inhibitor. Protein levels of caspase 3, caspase 9 and caspase 8 were increased in cells exposed to SYB in combination with an inhibitor of p53 expression in comparison with those of cells exposed to SYB alone (Fig. 6B and C). Inhibition

Table 1. The primers sequences of miR-34a and β -actin.

β -actin	F	5-TCCCTGGAGAAGAGCTACG-3'
	R	5-GTAGTTCCTGGATGCCACA-3'
miR-34a	Target sequence	5-UGGCAGUGUCUUAGCUGGUUGU 3'

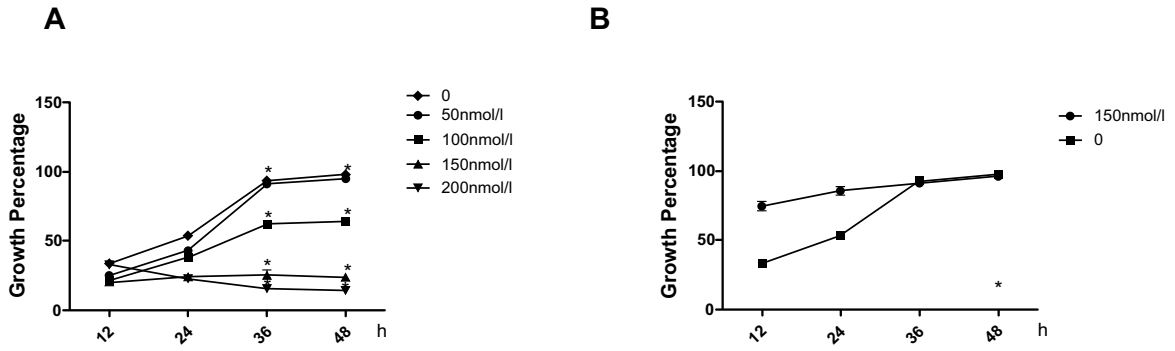


Fig (2). HepG2 and Hep3B cell (3000 cells/well, four replicates) growth percentage following exposure to SYB (final concentration: 50 nmol/L, 100 nmol/L, 150 nmol/L, or 200 nmol/L) for 12 h, 24 h, 36 h, and 48 h. A: HepG2 cell growth percentage, B Hep3B cell growth percentage. Presented as mean ± SD (n = 5). *Significantly different from the 12 h group (P < 0.05). Hep3B cells lacked P53 expression.

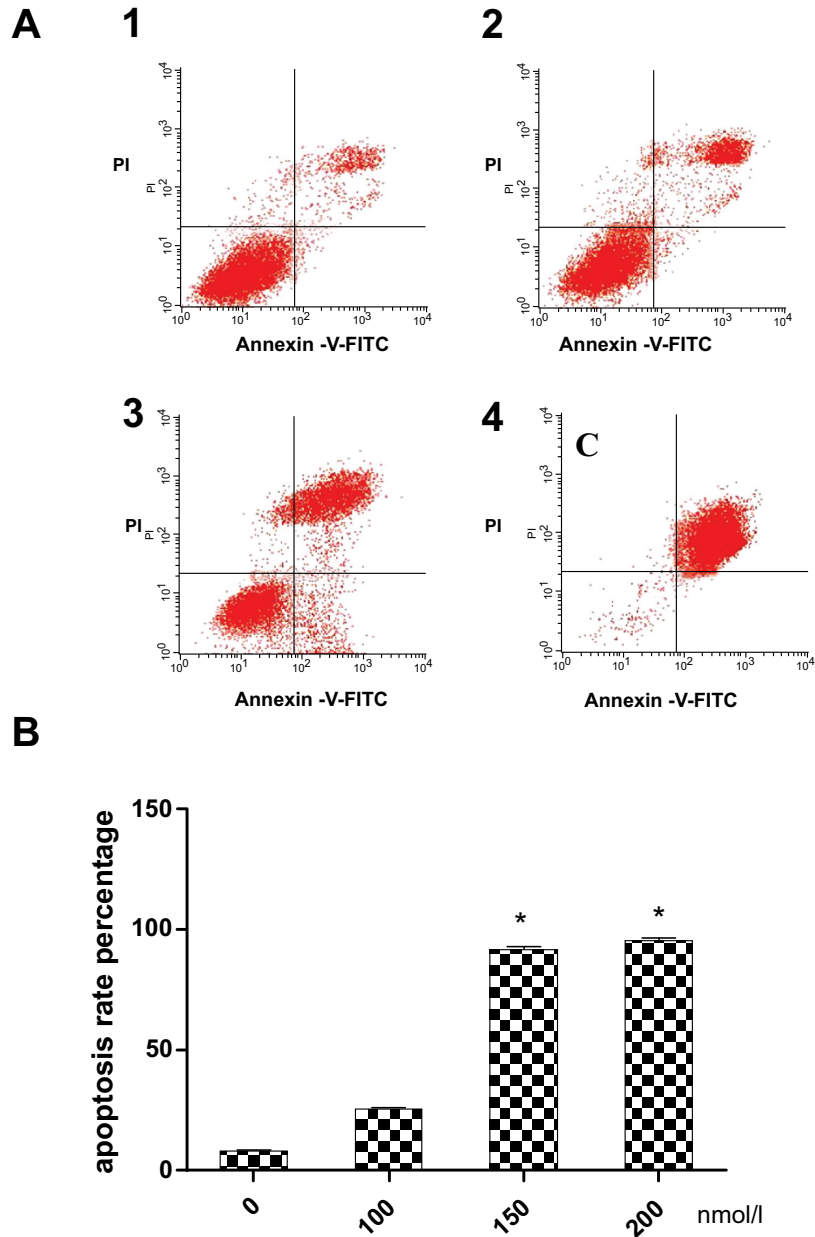


Fig (3). Annexin V/PI staining was used to detect HepG2 cell (1×10^6) apoptosis following exposure to SYB. A: Annexin V/PI staining. 1: 0 nmol/L; 2: 100 nmol/L; 3: 150 nmol/L; 4: 200 nmol/L. B: Percentage of apoptotic cells following exposure to different concentrations of SYB. X-axis, Annexin V; Y-axis, PI. Presented as mean ± SD (n = 5). * Significantly different from the 0 nmol/L group (P < 0.05).

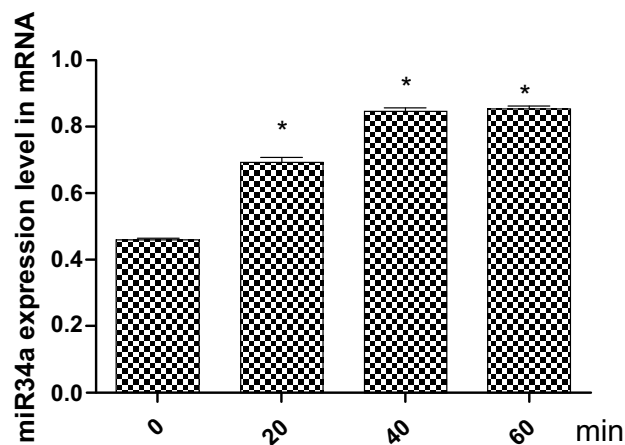


Fig (4). Relative miR-34a expression levels at 0, 20, 40 and 60 min following exposure to SYB. Presented as mean \pm SD (n = 5). *Significantly different from the 0 min group (P < 0.05). The mRNA level was calculated by the $2^{-\Delta Ct}$ method ($\Delta Ct = C_{t\text{sample}} - C_{t\beta\text{-actin}}$).

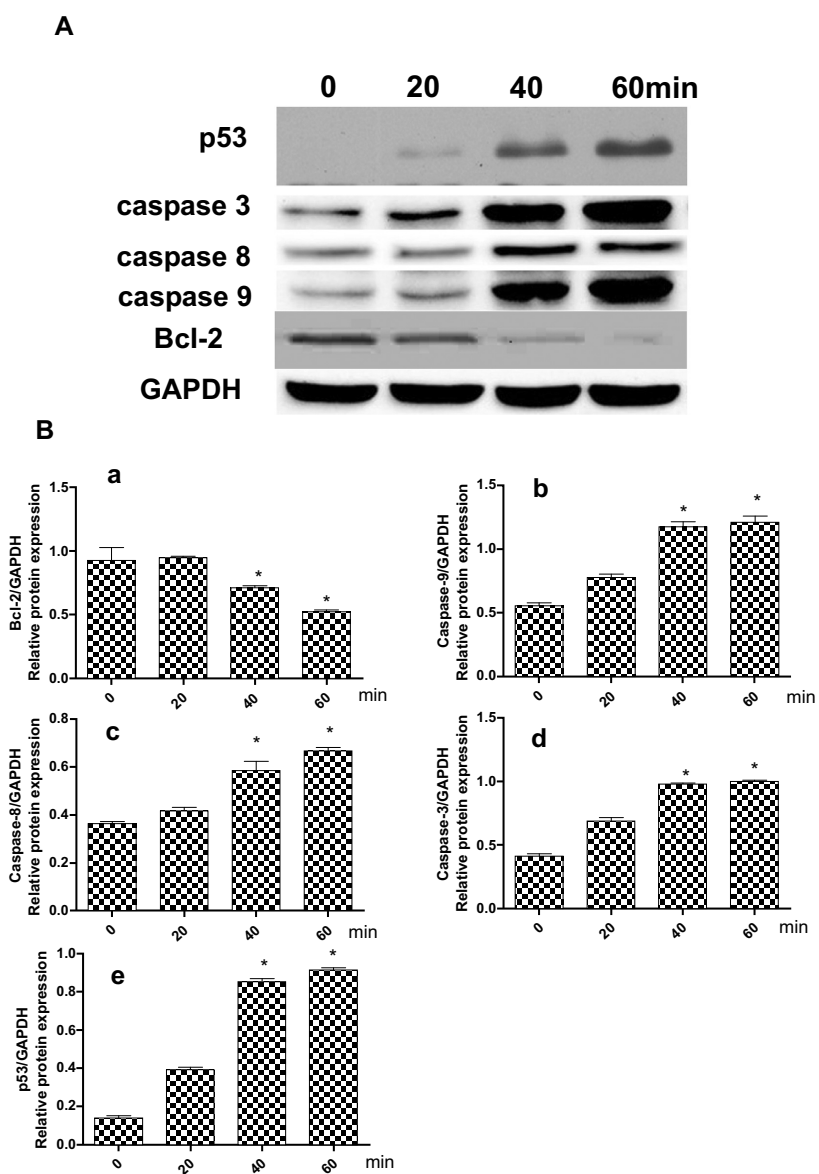


Fig (5). Protein levels of p53, caspase 3, caspase 9, caspase 8, and Bcl-2. A: expression of p53, caspase 3, caspase 9, caspase 8, and Bcl-2. B: Relative protein expression (protein/GAPDH) of p53, caspase 3, caspase 9, caspase 8, and Bcl-2. a: Bcl-2/GAPDH, b: caspase 9/GAPDH, c: caspase 8/GAPDH, d: caspase 3/GAPDH, e: p53/GAPDH. Presented as mean \pm SD (n = 5). *Significantly different from the 0 min group (P < 0.05).

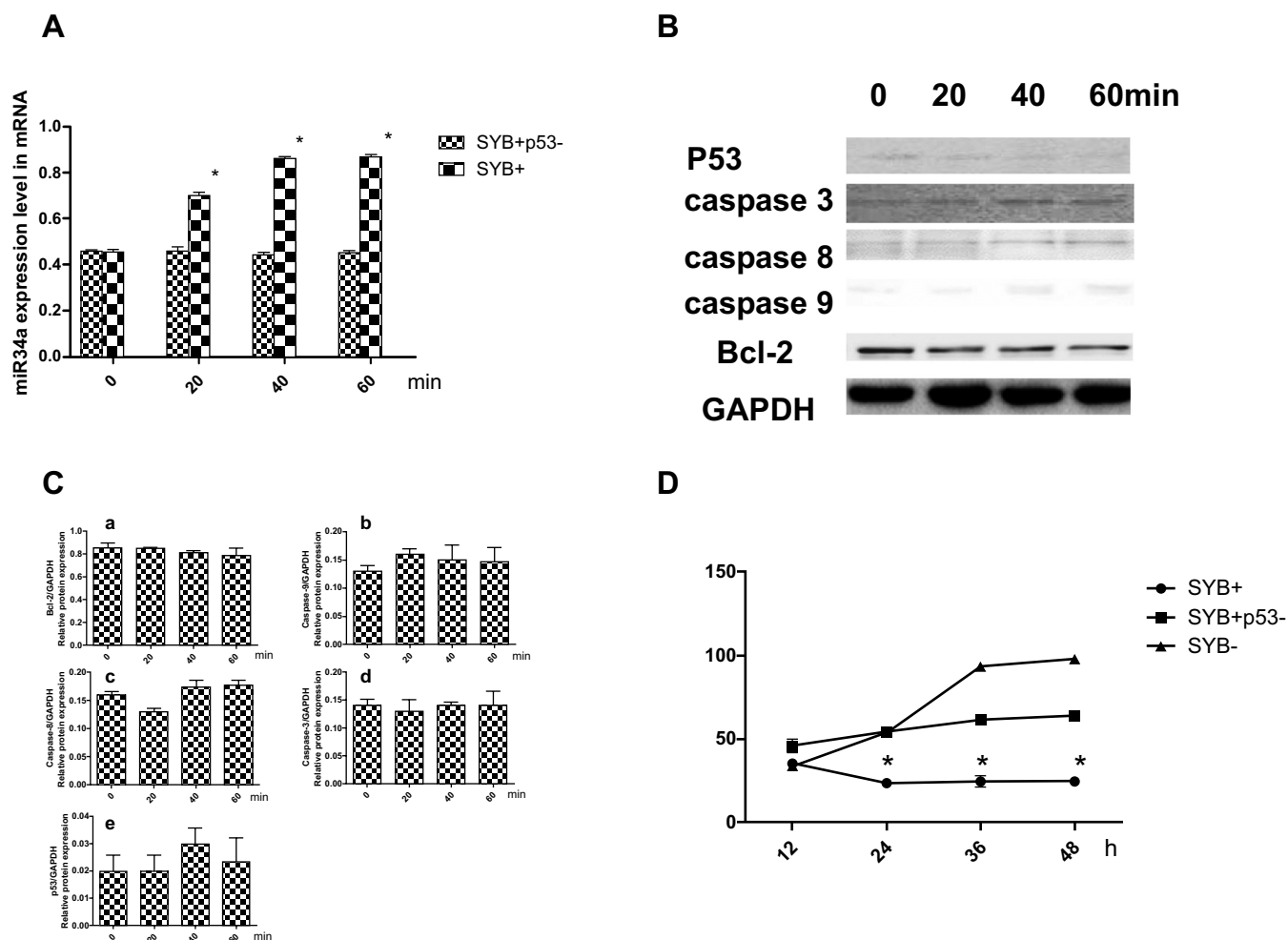


Fig (6). Influence of p53 inhibition on gene expression in HepG2 cells. A: Relative miR-34a expression levels were measured by qRT-PCR. B: Protein levels of p53, caspase 3, caspase 9, caspase 8, and Bcl-2. C: Relative protein expression (protein/GAPDH) of p53, caspase 3, caspase 9, caspase 8, and Bcl-2. a: Bcl-2/GAPDH, b: caspase 9/GAPDH, c: caspase 8/GAPDH, d: caspase 3/GAPDH, e: p53/GAPDH. Presented as mean \pm SD (n = 5). *Significantly different from the 0 min group (P < 0.05). D: HepG2 cell growth percentage at 12 h, 24 h, 36 h, and 48 h. *Significantly different from the 12 h group (P < 0.05).

of p53 expression promoted HepG2 growth in comparison with that of the control group (Fig. 6D).

To confirm the role of miR-34a in the inhibitory effect of SYB on HepG2 cell growth, miR-34a was silenced. In SYB-treated HepG2 cells lacking miR-34a expression, protein levels of p53 were increased in comparison with those of untreated cells that expressed miR-34a, whereas protein levels of caspase 3, caspase 9, caspase 8, and Bcl-2 were unchanged (Fig. 7B (a-e)). The growth rate of SYB-treated HepG2 cells lacking miR-34a expression was enhanced in comparison with that of untreated cells that expressed miR-34a (Fig. 7B (a-e)).

10. DISCUSSION

In a previous study, it was demonstrated that SYB is able to significantly attenuate brain injury induced by ischemia, and it was pointed out that SYB exerts neuroprotective effects against brain injury induced by ischemia by increasing the activities of antioxidant enzymes in the brain, thus decreasing free radical generation and improving mitochondrial function [19, 22]. However, the influence of SYB on liver cells has only been assessed in a few studies [19]. Recently, it has been shown that miR34a regulates p53 in HepG2 cells [23]. SYB protects hepatic cells against H₂O₂-induced oxidative stress through a mechanism that involves the AKT/Nrf2

pathway [19]. In this study, we showed that HepG2 cell apoptosis after SYB treatment was mediated by increased expression of p53 and miR34a during treatment. The concentration of SYB that most effectively inhibited HepG2 cell growth was 150 nmol/L. We also demonstrated that the inhibitory effect of SYB on HepG2 cell growth was mediated by miR-34a, which was highly expressed in HepG2 cells.

To gain further insight into the mechanism by which SYB inhibited HepG2 cell growth, (Fig. 7C) we evaluated signaling downstream of p53, revealing the probable role of p53 induction in miR-34a overexpression and down-regulation of Bcl2 leading to apoptosis. Previous reports have shown that P53 is a direct miR-34a target [23].

Our results suggest that SYB induced apoptosis by promoting expression of miR-34a. Indeed, miR-34a expression levels were correlated with the rate of apoptosis in HepG2 cells, in which tumor suppressor p53 activated miR-34a and down-regulated Bcl2. MicroRNAs are a class of critical regulators in cancer development and progression. Several microRNAs have been implicated in HCC; moreover, it has been recently reported that metformin alters the expression profile of microRNAs in many cancers [24]. microRNAs regulate the expression levels of a large proportion of genes, especially tumor suppressor genes and oncogenes such as p53 and

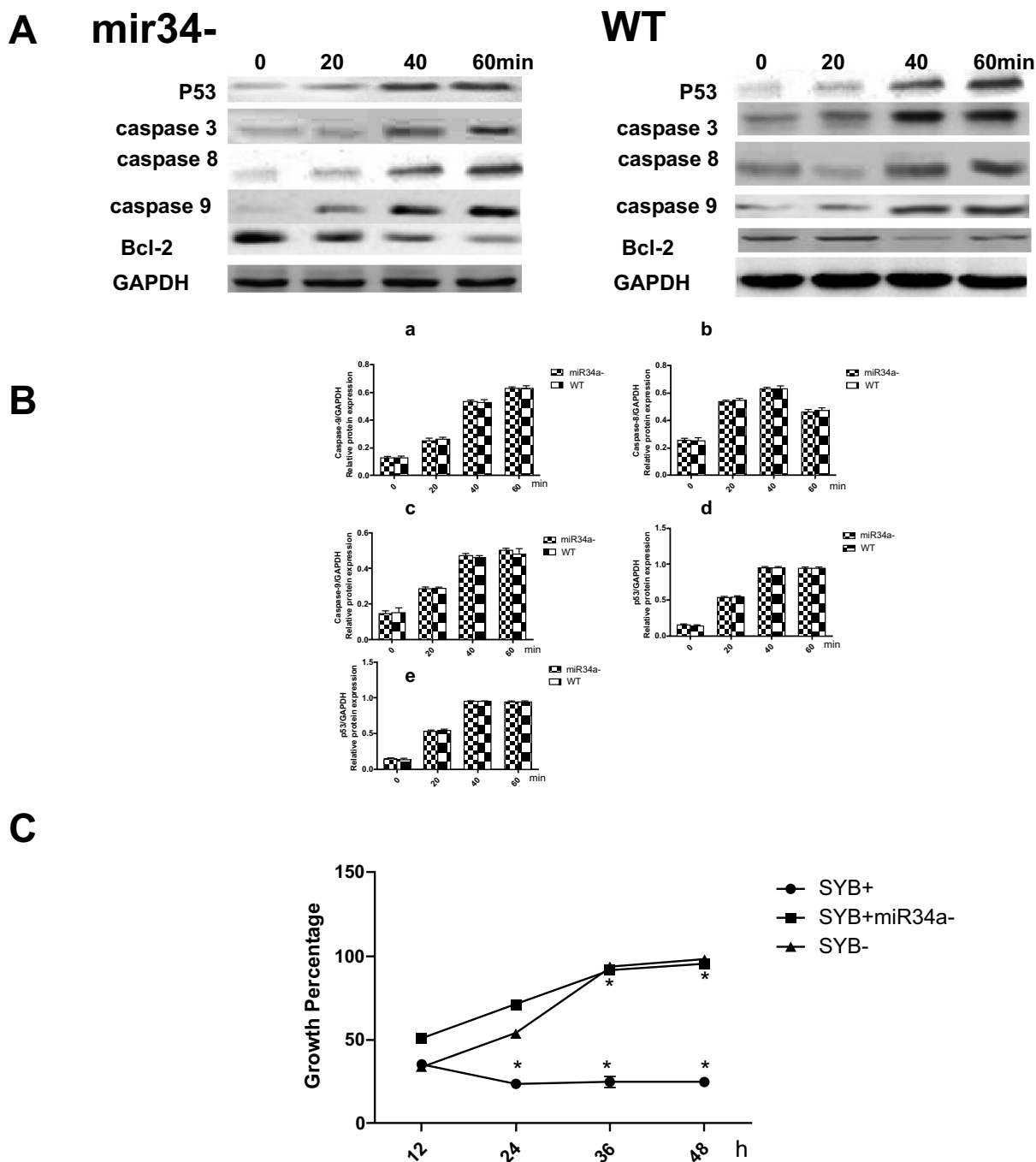


Fig (7). **A.** Influence of miR-34a inhibition on gene expression in HepG2 cells. **B. (a-e)** A-E: Protein levels of p53, caspase 3, caspase 9, caspase 8, and Bcl-2, and Relative protein expression (protein/GAPDH) of p53, caspase 3, caspase 9, caspase 8, and Bcl-2. Presented as mean ± SD (n = 5). *Significantly different from the 0 min group (P < 0.05). **C:** HepG2 cell growth percentage at 12 h, 24 h, 36 h, and 48 h. *Significantly different from the 12 h group (P < 0.05). WT: control without miR-34a inhibition.

Bcl2 [21]. Following expression of miR-34a/p53, caspase 9 was activated, after which apoptosis occurred *via* the mitochondrial pathway.

SYB represents a promising therapeutic that could be used clinically to activate apoptosis and inhibit cell division in liver cancer patients. Additional *in vitro* and *in vivo* studies are required to assess the effects of SYB against different cancers.

COMPLIANCE WITH ETHICAL STANDARDS

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Declared none.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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