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ORIGINAL ARTICLE

Rehmanniae Radix-Induced Apoptosis via Inhibition of PI3K/AKT/mTOR Signaling Pathways in Human Hepatocellular Carcinoma Cell Lines SMMC-7721

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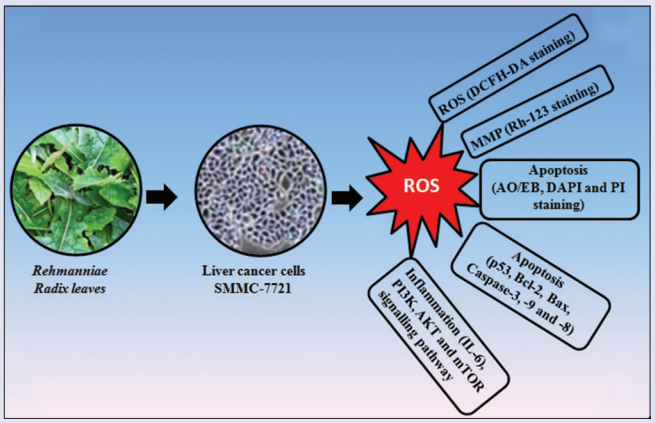
ABSTRACT

Background: Hepatocellular carcinoma (HCC) grades second among cancer-related deaths. *Rehmanniae Radix* (RR) has been exposed to have numerous pharmacological properties. **Objectives:** To inspect RR leaves extracts pro-apoptotic activities on SMMC-7721 liver cancer cell lines. **Materials and Methods:** Anti-cancer effect of RR extracts in SMMC-7721 liver cancer cell lines was aged rate of cell apoptosis (AO/EB, 4′,6-diamidino-2-phenylindole [DAPI], and PI staining), ΔΨm (Rh-123 staining), intracellular Reactive oxygen species (ROS) (DCFH-DA staining), quantitative polymerase chain reaction (mRNA expressions of Bax, p53, Bcl-2, caspase-3,-9,-8, IL-6, PI3K/AKT, and mTOR). **Results:** It has been shown that RR leaf ethanolic extracts suggestively declined cell viability, augmented the rate of cell apoptosis (AO/EB, DAPI, and PI staining), and caused the potential for ΔΨm, and induced over-accumulation of intracellular ROS (DCFH-DA staining) in SMMC-7721 cells after 24 h of exposure. In SMMC-7721 cell lines, we have examined anti-cancer activity to scrutinize the underlying mechanism by assessing the mRNA expressions of Bax, p53, Bcl-2, caspase-3,-9,-8, IL-6, PI3K/AKT, and mTOR. Results displayed the significant inhibition of the apoptosis as stated above related proteins. **Conclusion:** RR extracts exhibited its potential anti-cancer activity against SMMC-7721 cells by prompting apoptosis-induced cell death and inhibiting PI3K/AKT/mTOR signaling.

Key words: Cell signaling, hepatocellular carcinoma, *Rehmanniae radix*

SUMMARY

- RR extracts subdued the proliferation of SMMC-7721 cells.
- RR extracts tempted apoptosis in liver cancer cells.
- RR extracts induced cell death by triggering apoptosis in the HCC cells.
- RR extracts curbed the cell growth via PI3K/AKT/mTOR signaling pathways in SMMC-7721 cells.



Abbreviations used: HCC: Hepatocellular carcinoma; LC: Liver cancer; DMEM: Dulbecco modified eagle medium; RR: *Rehmanniae radix*; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; SDS: Sodium dodecyl sulfate; AO: Acridine orange; ROS: Reactive oxygen species; PI: Propidium iodide; EB: Ethidium bromide; Rh-123: Rhodamine 123; DCF: Dichlorofluorescein; PBS: Phosphate-buffered saline; DAPI: 4′,6-diamidino-2-phenylindole.

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INTRODUCTION

Liver Cancer (LC) or Hepatocellular Carcinoma (HCC) ranks second amid the most cancer-related deaths.[1] According to WHO reports, more than 1 million deaths from HCC would upsurge by 2030.[2] After pancreatic cancer; it ranks second most deadly cancer with 18% of average survival rate as per the reports of Siegel *et al.*[3] The LC death augmented by 43% from 2000 to 2016 in the USA.[4] The prevalence of HCC is more mutual in people with risk factors such as hepatitis virus infection, cirrhosis, alcoholism, and smoking. The age-related occurrence of HCC amplified from 1.6/100,000 individuals to 4.6/100,000 persons among the different American ethnic.[5] Administration of vaccines and antiviral agents against HBV and HCV brought variations in the etiology of the disease. Obesity and nonalcoholic fatty liver syndrome have become essential factors influencing the increase of LC deaths in Western countries.[6] Although numerous spreads in the treatment of HCC have been improved and cases controlled through liver transplantation remains low. Although chemotherapy and radiotherapy have valuable effects, their application is limited due to their adverse effects.[7] The higher invasive nature and metastatic nature of cancer with higher relapse rates increase the difficulties in treating the disease. Hence, there is a need for substitute therapeutic modalities for effective treatment of LC.[8]

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Rehmanniae Radix (RR) is a perennial herb with latent pharmacological properties such as antioxidant, anti-inflammation[9], proinflammatory, antidiabetic[10], and neuroprotective properties.[11] Development of CT26 carcinoma and B16 melanoma was effectually inhibited by RR plant in C57BL/6 and BLAB/c mice, respectively, by inducing tempts toll-like receptor-4 mediate anti-cancer immunity.[12] In *in vitro* and *in vivo* studies displayed marked production in the levels of proinflammatory cytokines.[13] It also presented inhibitory effects against Yac cells by inducing natural killer cells and against CT26 lung cancer.[12] Cheng *et al*. testified that the RR exhibits strong anti-tumor cell proliferation action and induces apoptosis on bone cancer MG-63 cells through enlarged production of reactive oxygen species (ROS).[14] Hence, it is imagined that this plant may have budding anticancer activity against LC.

The contemporary work purposes to measure the anticancer activity of *RR* leaves extracts against SMMC-7721 hepatic cancer cell lines as a potential candidate for LC.

MATERIALS AND METHODS

Chemicals

All the chemical reagents employed in the study were obtained from Sigma Aldrich Chemicals (USA). Dyes applied in the study were acquired from Hi-Media (USA).

Cell culture

Human HCC SMMC-7721 cells plated in Dulbecco modified eagle medium (DMEM) medium with 10% FBS and 100 µg/mL streptomycin. The cells were incubated in 5% CO_2 at 37°C.

Plant material and extract preparation

The RR leaves were composed from the Affiliated Hospital of North Sichuan Medical College (China) and the leaves were splashed and shade dried at 27°C ± 2°C and grounded. Using a Soxhlet apparatus, about 300 g of the plant materials was extracted with 1 L ethanol at 60°C–80°C for 8 h and filtered. After solvent evaporation, the extracts were arranged in different concentrations (50, 100, 200, 250, 300, 350, and 400 µg/mL) for the study.

Cell viability assay

The cytotoxic effects of RR leaf extract were considered using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. About 5×10^5 cells/well cells were sowed to the 96-well plate and incubated with numerous concentrations (50–400 µg/mL) RR extracts for 48 h. Approximately 20 µL of 5 mg/mL of MTT solution was administered to the cell culture, and the formazan (purple crystals) produced were liquefied in 150 µL of DMS read at 490 nm. Out of the results, the IC_{50} values were measured using linear regression analysis.

Extracellular reactive oxygen species by DCFH-DA staining

Evaluation of the production of ROS in HCC SMMC-7721 cells was accomplished with DCFH-DA staining, which can be oxidized with fluorescent dichlorofluorescein (DCF) due to ROS formed inside the cell. Before treated cells with different concentrations of RR extract (200 and 250 µg/mL), about 1×10^6 cells/well were inoculated to 6-well plates incubated for 24 h. Besides, the cells were treated with 10 µL of DCFH-DA for 2 h. Fluorescence was recited at 530 nm by a fluorescence microscope (Nikon, Eclipse TS 100, Japan).

Δψm analysis by Rh-123 staining

The Δψm is a symbol of early-stage apoptosis that can be evaluated using the Rh-123 staining. The cells were seeded in 6 well plates in DMEM and treated with DMSO as control, 200 and 250 µg/mL of RR leaf extracts in DMEM medium. About 10 µg/mL of Rh-123 was added and incubated for 30 min at 37°C. Furthermore, the cells were wetted with phosphate-buffered saline (PBS) and detected using a 485–545 nm fluorescence microscope (Nikon, Eclipse TS 100, Japan).

AO/EB, 4′,6-diamidino-2-phenylindole and propidium iodide staining

About 5×10^5 cells were imperiled to trypsinization and added to well plates. After being treated with different RR extracts concentrations (200 and 250 µg/mL), the cells were incubated for 24 h, followed by fixation in CH_3OH: CH_3COOH (3:1) for 30 min at 4°C. The RR extracts treated cells were washed by cold PBS and stained with AO/EB (1:1 ratio) for 5 min. The reaction mixture was initiated under a fluorescence microscope at 40x magnification with an excitation filter set at 510–590 nm.

For 4′,6-diamidino-2-phenylindole (DAPI) staining, overnight grown SMMC-7721 cells were added to the six-well plates. Different concentrations of RR leaves extract of 200 and 250 µg/mL were administered to the cells. Then, the cells 4% paraformaldehyde fixated and exposed to permeabilization using 0.1% Triton X-100. Finally, the slides with cells were stained DAPI and pragmatic using a fluorescence microscope.

The formation of apoptotic nuclei in the cancer cells can be measured using PI staining. Different concentrations of RR extracts of 200 and 250 µg/mL and incubated for 24 h. The cells were subjected to trypsinization and fixed with paraformaldehyde, and treated with 1 mg/mL of PI followed by PBS washing. Finally, they were detected under a fluorescence microscope.

Real-time polymerase chain reaction technique

The cell signaling regulators mRNA expressions, i.e., p53, Bax, caspase-3, caspase-8, caspase-9, Bcl-2, IL-6, PI3K, AKT, and mTOR contributing to cancer pathogenesis, were examined using quantitative real-time polymerase chain reaction (qRT-PCR). About 3×10^5 cells are cultured until they influence 80% confluence. Further, They were treated with RR extracts at 200 and 250 µg/mL for 24 h. The total RNA extraction (Qiagen RNeasy Mini Kit) was completed as per the manufacturer protocol, and cDNA was formed using a reverse transcription system (Bio-Rad S1000 Thermocycler) and stored at -20°C [Table 1]. The qRT-PCR reaction was finished using commercial PCR Kits (Qiagen). Each reaction was ended in triplicates, and the Δct method was employed to classify the fold changes.

Statistical analysis

The data were articulated as mean ± SEM Graph pad prism 6.01 employed for Statistical analysis with ANOVA followed by Tukey's *post hoc* test analysis was used. $P < 0.05$ were measured significant.

RESULTS

Cytotoxic effects of *Rehmanniae radix* extracts analysis through 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide dye

The cytotoxic and anti-proliferative effects of RR extracts were evaluated using an MTT assay. It was detected that RR extracts repressed SMMC-7721 cells proliferation with growing concentration (50–400 µg/mL). Significant inhibition was perceived in the treated cells

[Figure 1]. Compared to the controlled cells, the dose of 200 µg/mL was designated for further examination. The IC_{50} value was 250 µg/mL.

Effect of *Rehmanniae radix* extracts on intracellular reactive oxygen species generation by *Rehmanniae radix* extracts

ROS generation levels in the control and RR extracts treated SMMC-7721 cells were determined after 24 h incubation [Figure 2a]. The intracellular ROS level was remarkably augmented in the RR extracts treated cells compared to the controls when treated at 200 and 250 µg/mL. Results exposed a significant rise in the ROS levels detected under the fluorescence microscope [Figure 2b].

Effect of *Rehmanniae radix* extracts on Δψm analysis by Rh-123 staining

Δψm activity judged by Rh-123 staining exhibited a marked diminution in the accumulation of Rh-123 staining inside the cells, representing the depletion of the membrane potential in the mitochondria with an increase in the concentration of RR leaves extracts (200 and 250 µg/mL) as shown in Figure 3a. Dissipation of the membrane potential was an important factor in persuading apoptosis in the cancer cells [Figure 3b].

Effect of RR extracts induced apoptotic changes in LC SMMC-7721 Cells by AO/EB, DAPI, and PI staining.

In general, AO/EB, DAPI, and PI staining estimated apoptotic variations in the cells. Clear apoptotic structures were pragmatic in the extract-treated cells. In AO/EB staining, the cells were compared to controls liable on their apparent fluorescent coloration [Figure 4a]. The untreated cells exhibited a bright green color exhibited

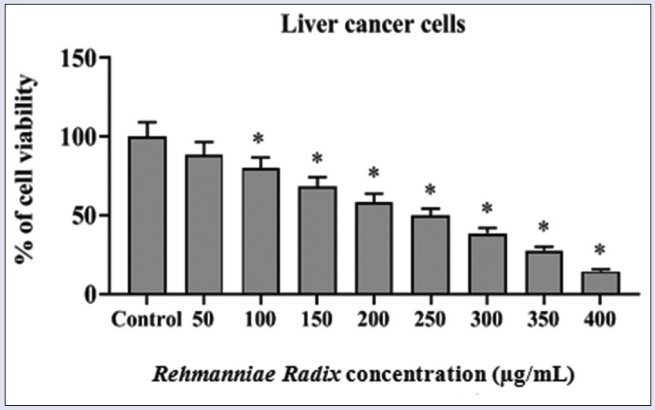

Figure 1: Effect of rehmanniae radix extracts on cell viability in SMMC-7721 LC cell lines. The values are given as mean ± SD of three experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from control: $^{*}P < 0.05$

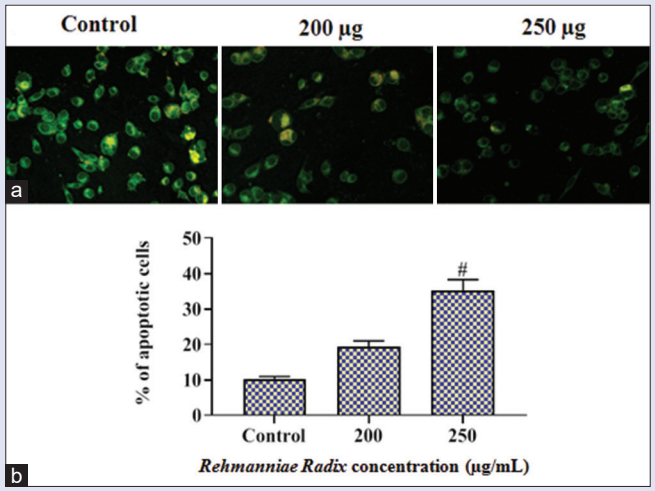

Figure 3: Effect of rehmanniae radix extracts on Δψm in SMMC-7721 cells. a) Rehmanniae radix extract (200 and 250 µg/mL) shown the apoptotic cells in green fluorescence. The values are given as mean ± SD of three experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from control: $^{\#}P < 0.05$

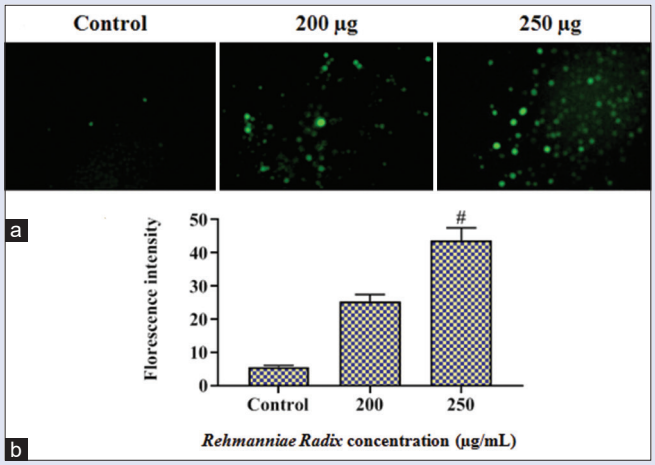

Figure 2: Effect of rehmanniae radix extracts on ROS generation in SMMC-7721 HCC cell lines. (a) After 24 h incubation, rehmanniae radix 200 µg and rehmanniae radix 250 µg treated cells indicated ROS generation. (b) The values are given as mean ± SD of three experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from control: $^{\#}P < 0.05$

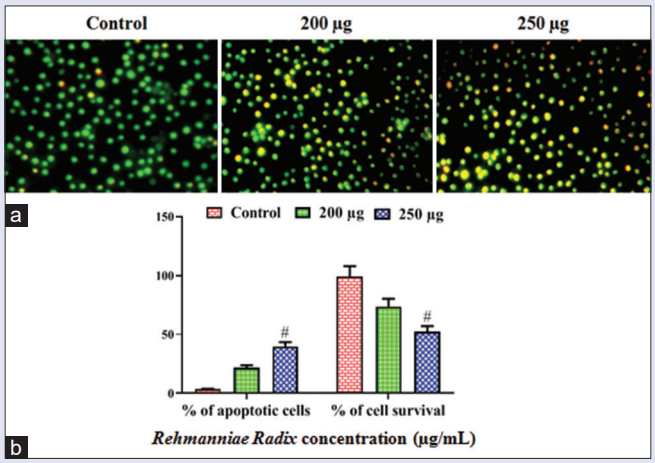

Figure 4: Effect of rehmanniae radix leaves extracts induced apoptotic morphological damages in LC cells (a) LC cells treated within vehicle control and rehmanniae radix leaves extracts at various concentrations (200 & 250 µM) at 24 h, stained with dual dye EB/AO and then analyzed by fluorescence microscopy. (b) Percentage of apoptotic cells were calculated by scoring apoptotic and viable cells. The values are given as mean ± SD of three experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from control: $^{*}P < 0.05$

intact nuclear structure, indicating viable cells, whereas, in RR extracts, treated cells disclosed a decrease in the number of green cells with the upsurge in orange at the concentrations of 200 and 250 µg/mL, as per Figure 4. Results show the cells underwent cell death via apoptosis [Figure 4b]. The DAPI staining results were gauged as disruption of cell structure and nucleus, indicating the cells' morphological conditions [Figure 5a]. After the incubation, the control cells continued intact, whereas the RR extract-treated cells exhibited noticeable changes inducing apoptosis [Figure 5b]. In addition, PI staining showed a clear sign of disruption of cell and nucleus structure, as in Figure 6a. The RR leaf extracts-treated cells showed signs of activating apoptosis [Figure 6b].

Effect of *Rehmanniae radix* extracts on mRNA expressions of apoptosis mediators

The mRNA expression levels of the apoptotic regulators were measured by qRT-PCR analysis. The mRNA expressions of the mediators such as p53, Bax, caspase-3,-8, and-9 [Figure 7]. The results obviously showed the obvious upsurge in the aforesaid apoptotic mediators' expression levels when treated with RR extracts at the concentration of 200 and 250 µg/mL. The results showed a 0.5-fold increase in p53, Bax, and caspase-8, whereas a fold increase was detected in the levels of caspase-3, and-9 compared to the controls. Furthermore, the results recommend that RR leaves extracts induced cell death by triggering apoptosis in the HCC cell lines.

Effect of *Rehmanniae radix* leaves extracts on PI3K/AKT/mTOR signaling

The mRNA expression levels of the apoptosis-related genes regulating the downstream effectors were weighed using RT-PCR. The results showed noteworthy inhibition in the mRNA expression levels of Bcl-2, IL-6, PI3K, AKT, and mTOR signaling pathway [Figure 8]. The results disclosed a 0.75-fold reduction in Bcl-2, a 0.5-fold decrease in IL-6 and mTOR, and a 0.25-fold diminution in PI3K and AKT levels compared to control. Thus, the results approve the inhibition of cell proliferation, thereby promoting apoptosis.

DISCUSSION

HCC, standing second among the most cancer-related deaths, is a considerable threat. Although chemotherapeutic treatments were

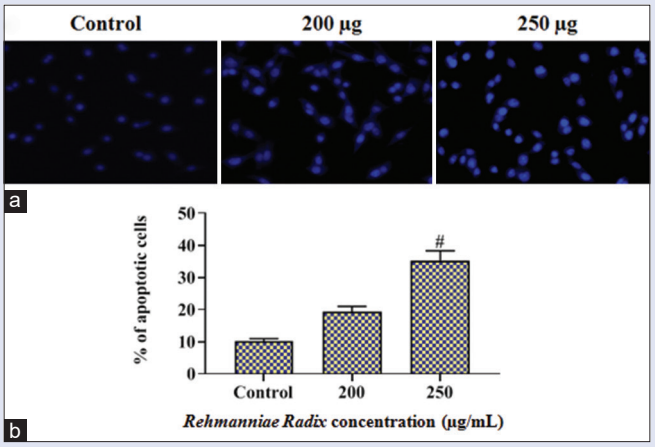

Figure 5: Effect of rehmanniae radix leaves extracts induced nuclear morphological damages in LC cells. (a) LC cells treated within vehicle control and rehmanniae radix leaves extracts at various concentrations (200 & 250 µg) at 24 h, stained with DAPI dye and then analyzed by fluorescence microscopy. (b) Percentage of apoptotic cells nuclear stages were calculated by scoring apoptotic and viable cells. The values are given as mean± SD of six experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from vehicle control: $^{*}P < 0.05$

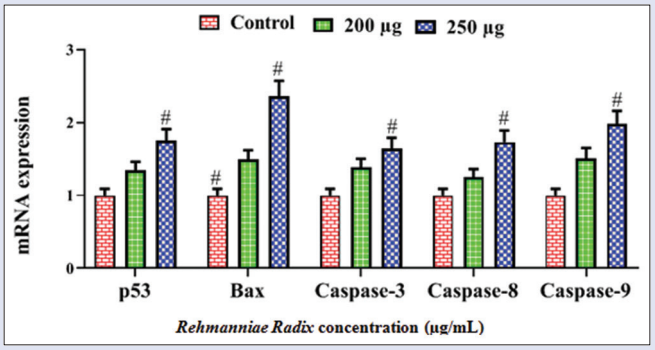

Figure 7: Effect of rehmanniae radix extracts on apoptosis-related protein mRNA expressions in SMMC-7721 LC cell lines. The values are given as mean ± SD of three experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from control: $^{\#}P < 0.05$

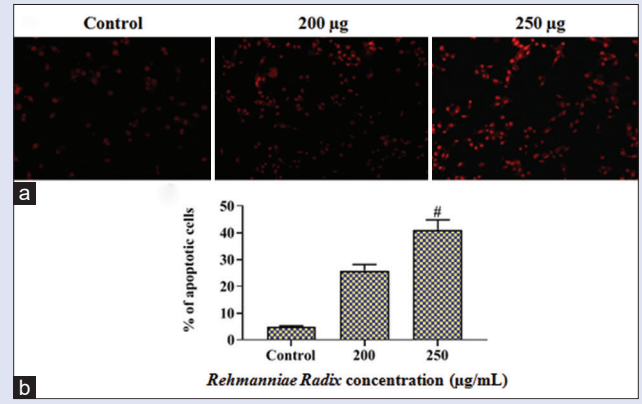

Figure 6: Effect of rehmanniae radix extracts induced apoptosis by DAPI staining in SMMC-7721 cells. (a) Cells were treated with rehmanniae radix extracts (200 and 250 µg/mL) and incubated for 24 h. (b) The values are given as mean ± SD of three experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from control: $^{\#}P < 0.05$

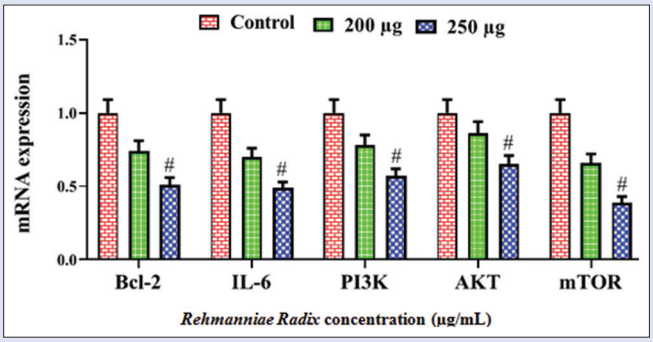

Figure 8: Effect of rehmanniae radix extracts on mRNA expressions of apoptosis-related protein in SMMC-7721 hepatocarcinoma cell lines. The values are given as mean ± SD of three experiments in each group ANOVA followed by DMRT. Asterisks indicate statically different from control: $^{\#}P < 0.05$

Table 1: Forward and reverse primers used in gene expression analysis

Gene	Forward	Reverse
Bax	5'-GTTTCATCCAGGATCGAGCAG-3'	5'-CATCTTCTTCCAGATGGTGA-3'
p53	5'-CCCCTCCTGGCCCCTGTCATCTTC-3'	5'-GCAGCGCCTCACAACCTCCGTCAT-3'
Caspase-3	5'-TGGCCCTGAAATACGAAGTC-3'	5'-GGCAGTAGTCGACTCTGAAG-3'
Caspase-8	5'-AATGTTGGAGGAAAGCAAT-3'	5'-CATAGTCGTTGATTATCTTCAGC-3'
Caspase-9	5'-CGAACTAACAGGCAAGCAGC-3'	5'-ACCTCACCAAATCCTCCAGAAC-3'
Bcl-2	5'-CCTGTGGATGACTGAGTACC-3'	5'-GAGACAGCCAGGAGAAATCA-3'
IL-6	5'-CCAGCTATGAACTCCTTCTC-3'	5'-GCTTGTTCCTCACATCTCTC-3'
PI3K	5'-CTCTCCTGTGCTGGCTACTGT-3'	5'-GCTCTCGGTTGATTCCAAACT-3'
AKT	5'-GGACAAGGACGGGCACATTA-3'	5'-CGACCGCACATCATCTCGTA-3'
mTOR	5'-CTGGGACTCAAATGTGTGCAGTTC-3'	5'-GAACAATAGGGTGAATGATCCGGG-3'
β-actin	5'-GTGACATCCACACCCAGAGG-3'	5'-ACAGGATGTCAAAACTGCCC-3'

IL: Interleukin

available, an alternate effective drug was wanted to reduction the toxic side effects.[15] RR leaves extracts are a perennial herb that has been used as an antipyretic, cardiac stimulator, hemostasis, and detoxification. MTT assay was employed to evaluate the anti-proliferative effect of HCC SMMC-7721 cells offered marked cytotoxic effects from 200 μg/mL and caused cell death in the LC cells at an IC_{50} value of 250 μg/mL.

Mitochondria was known to be the chief intracellular site for the production of ROS.[16] In general, ROS levels have augmented in cancer cells. Amplified ROS levels can induce apoptosis of cells thus mitochondria-targeting can give cancer-specific therapy.[17] Intracellular ROS generation inside the cell evaluated by DCFH-DA staining showed increased accretion of ROS inside the HCC when treated with 200 and 250 μg/mL, demonstrating apoptosis in cancer cells.

Δψm dissipation is the dangerous factor in activating apoptosis through sequential caspase activation. Production of ROS causes the Δψm appraised by Rh-123 staining.[18] However, whereas the bright green color exhibited by the LC SMMC-7721 cells presented a manifest surge of ROS with depletion in the mitochondrial potential when rested with RR extracts for 24 h. Variations in the morphological structures due to apoptosis in SMMC-7721 cells were judged using AO/EB, DAPI, and PI staining. Apoptosis is a natural phenomenon in the cell cycle, keeping the cell population in hemostasis.[19] Apoptosis may include cell shrinkage, dissipation of the cell membrane, mitochondrial membrane potentials, DNA fragmentation, and chromatin condensation.[20] AO/EB staining displayed marked apoptosis by showing orange stained apoptotic structures compared to the controls viewing intact structures. Loss in cell structure with nuclear condensation was detected in the DAPI staining. The two staining PI staining disclosed chromatin condensation with structural loss, signifying the generating of apoptosis in LC cells.

Cell apoptosis is a complex process with multiple signaling pathways where caspases play a crucial role in apoptosis regulation, which can also be activated by p53.[21] Caspase-3 is a decisive regulator activating apoptosis by two signaling cascades; death receptor pathway and the mitochondria-mediated pathway linking caspase-8,-10, and-9, respectively.[22,23] RT-PCR analysis after RR extract treatment presented a 0.5-fold increase in p53, Bax, and caspase-8, whereas a fold upsurge was detected in the levels of caspase-3 and-9 compared to the control. Hence, the results propose the apoptotic regulators' involvement and settle the apoptosis-induced cell death in SMMC-7721 cells.

To appreciate RR extracts mechanism behind the anticancer activity, the critical apoptotic regulators such as Bcl-2, IL-6, PI3K, AKT, and mTOR were analyzed using qRT-PCR analysis. The PI3K/AKT pathway was revealed to be hardier to chemotherapy with an aggressive phenotype.[24,25] Numerous correlations have been found between the PI3/AKT pathway and cell survival, which portrays it as a potential target for LC therapy.[26] The results displayed that RR leaves extract-treated cells marked reduction in the Bcl-2, IL-6, PI3K, AKT, and mTOR expression levels, which further established the involvement of, PI3K, AKT, and mTOR inhibition contributing to the anticancer activity of RR extracts.

CONCLUSION

Our results evidently showed that the RR extracts have latent anti-cancer activity against HCC cell lines SMMC-7721 by inducing caspases mediated apoptosis through mitochondrial signaling pathway and inhibiting PI3K, AKT, and mTOR pathway. Hence, it settles that RR leaf's extracts are a budding candidate for LC.

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Conflicts of interest

There are no conflicts of interest.

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