Pharmacogn. Mag.
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ORIGINAL ARTICLE

Anti-cancer and Cell Toxicity Effects of Royal Jelly and its Cellular Mechanisms against Human Hepatoma Cells

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Submitted: 15-Jan-2022 Revised: 20-Feb-2022 Accepted: 27-Apr-2022 Published: 19-Sep-2022

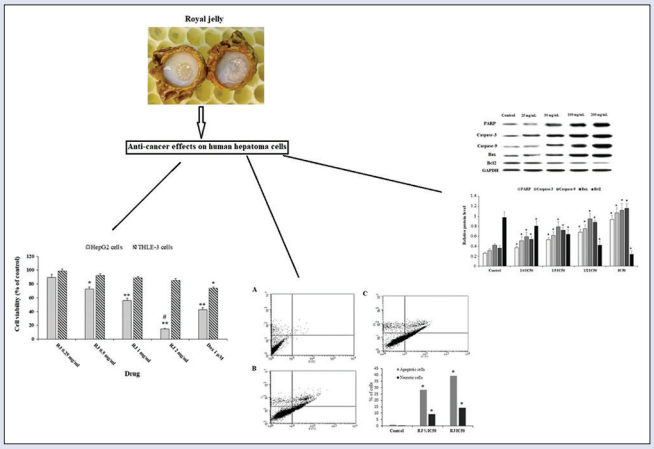
ABSTRACT

Background: This investigation was planned to evaluate the anti-cancer effects of royal jelly (RJ) obtained from *Apis mellifera* compared with doxorubicin as an anthracycline with potent anti-cancer activity and its cellular mechanisms against the human hepatoma cell line HepG2. **Materials and Methods:** The cytotoxic effects of various concentrations of RJ on the HepG2 cell viability by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay were studied. For the primary and late apoptosis in HepG2 cells exposed with RJ, we used the Annexin-V (AV) assay using cytometry analysis using the commercial kit as explained by the manufacturer's guidelines. Real-time polymerase chain reaction assessed the gene expression of miRNA-34a (miR-34a), caspase-3, Bcl-2, and Bax. We also used the western blot to evaluate the protein expression levels of poly (ADP-ribose) polymerases (PARP), Caspase-3, Caspase-9, Bcl-2, and Bax. **Results:** The IC_{50} value of RJ was found 1.13 mg/mL for HepG2 cells. RJ revealed no cytotoxicity on normal THLE-3 cells with IC_{50} >2 mg/ml. RJ at the concentrations of ½ IC_{50} significantly increased ($p < 0.05$) apoptotic and necrotic cells from 0.96% to 28.3% and 9.3%, respectively. RJ at the concentration of IC_{50} significantly increased ($p < 0.05$) apoptotic and necrotic cells from 0.96% to 39.2% and 14.12%, respectively. The expression of miR-34a, Caspase-3, and the Bax gene was considerably ($p < 0.001$) up-regulated as they are dose-dependent, whereas the expression level Bcl-2 was considerably ($p < 0.05$) declined in the HepG2 cells exposed with RJ. Treatment of HepG2 cells treated with RJ triggered a significant inhibition of Bcl-2 protein, whereas a significant rise in PARP, Caspase-3, Caspase-9, and Bax expression was observed. **Conclusion:** Our results showed the promising anti-cancer effects of RJ against HepG2 cells, whereas the induction of apoptosis by various pathways is considered the main mechanism underlying the cytotoxic effect of RJ against HepG2 cells. The present study's findings propose that RJ can be a candidate agent for treating human HCC.

Key words: Anti-cancer, apoptosis, cytotoxicity, natural products, royal jelly

SUMMARY

- We evaluated the anti-cancer effects of RJ and its cellular mechanisms against the human hepatoma cell line HepG2.
- The IC_{50} value of RJ was found to be 1.13 mg/mL for HepG2 cells. RJ revealed no cytotoxicity on normal THLE-3 cells with IC_{50} >2 mg/ml.
- RJ induced the apoptosis by various pathways in HepG2 cells.
- RJ can be a candidate agent for treating of human HCC.



Abbreviations used: ATCC = American Type Culture Collection, Bcl-2 = B-cell lymphoma-2, DOX = Doxorubicin, FITC = Fluorescein isothiocyanate, GADPH = Glyceraldehyde 3-phosphate dehydrogenase, HCC = Hepatocellular carcinoma, mir-34a = Mirna-34a, PMSF = Phenylmethylsulfonylfluoride, PVDF = Polyvinylidene difluoride, PI = Propidum iodide, RJ = Royal jelly, SDS-PAGE = Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, TBST = Tris-buffered saline Tween-20, PARP = Poly (ADP-ribose) polymerases, HRP = Horseradish peroxidase, ROS = Reactive oxygen species, AV = Annexin-V, FITC = Fluorescein isothiocyanate, Bax = Associated X-protein

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DOI: 10.4103/pm.pm_18_22

Access this article online
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INTRODUCTION

Hepatocellular carcinoma (HCC) is a principal liver cancer that initiates from hepatocyte cells in the liver. HCC is the second most frequent cause of cancer death worldwide,[1] whereas the disease is considered the fifth most common cancer, which annually influences more than 1 million people worldwide.[2] Considering the risk factors of HCC, previous studies demonstrated some factors such as transgenic oncogenes, excessive alcohol consumption, latent viral infection, iron overload, and so on.[3]

At present, treatment of HCC is clinically tricky. Several therapies including chemotherapy with chemical agents, radiotherapy, surgery, and so on have been reported to recover the prognosis and long-term survival of patients.[4] Concerning chemotherapy treatment of HCC,

Cite this article as: Alnomasy SF, Al Shehri ZS. Anti-cancer and cell toxicity effects of royal jelly and its cellular mechanisms against human hepatoma cells. Phcog Mag 2022;18:635-40.

there are numerous synthetic drugs such as methotrexate, doxorubicin, cisplatin, taxis, and so on.[5] However, recent investigations have described various restrictions and side effects in the use of synthetic agents such as bone marrow suppression, fatigue, nausea, vomiting, hair loss, and so on and the expressions of multi-drug resistance genes.[5,6] These factors render the researchers new anti-cancer agents, particularly in natural products with higher effectiveness and minimum toxicity.

Natural products are recognized as a respected source of suitable new agents with an infinite chemical diversity detected in millions of species of herbs, animals, marine organisms, and microbes.[7] Recently, increasing interest has been observed in useful food materials and proper diet to treat and prevent numerous diseases such as cancers.[8] Among the beneficial foods, products from the beehive, such as royal jelly (RJ), honey, and propolis, are well known for having health-promoting possessions.[9]

RJ as a beneficial nutritious substance is secreted from the hypo-pharyngeal and mandibular salivary glands of young nurse honey bees.[10] Because of the high content of different bio-active metabolites such as polyphenols, proteins, lipids, carbohydrates, and mineral salt, RJ displayed numerous pharmacological characteristics such as antioxidant, anti-inflammatory, anti-cancer, neurotrophic, anti-diabetic, anti-microbial, and so on.[11] This survey was planned to assess the anti-cancer effects of RJ obtained from *Apis mellifera* compared with doxorubicin (DOX) as an anthracycline with potent anti-cancer activity and its cellular mechanisms against the human hepatoma cell line HepG2.

MATERIALS AND METHODS

Royal jelly

Fresh RJ was provided from June 2021 from Langstroth hives containing larvae of 3-day-old queen bees (*A. mellifera*) from beekeepers of Riyadh, Saudi Arabia. Samples had a butter-like appearance and a yellowish color with a somewhat sour flavor. After dissolving the RJ in normal saline, the suspension was filtered through a filter paper (Whatman membrane, England).

Secondary metabolite contents

The total amount of phenol in the RJ specimen was studied as previously defined by Singleton *et al.*,[12] and the content was displayed as mg gallic acid equivalents (GAE)/g. The amount of flavonoid content in the RJ specimen was calculated as defined by El-Guendouz *et al.*,[13] and contents were exhibited as milligrams of quercetin (Sigma-Aldrich, St. Louis, MO) equivalents per gram of RJ (mg QE/g RJ). The protein content in the RJ specimen was calculated based on the Bio-Rad assay; the content was finally described as a percentage (%) through bovine serum albumin (Sigma-Aldrich, St. Louis, MO).[14]

Evaluation of moisture and ash contents

The content of the RJ sample was determined after incubation of the sample (1g) in an oven at 550°C for 8 hours. After this time and when the sample temperature was appropriate, the remaining container was weighed and the amount of ash was displayed as percent in 1 g RJ.

The moisture content of the RJ sample was measured by incubating one gram of the RJ sample at 105°C for 3 hours and weighing the sample after equilibrating its temperature. The specimen was kept again in the oven for 60 minutes to check if the weights declined. This procedure was repeated until completing the water evaporation with a stable weight.[15]

Cell culture

The HepG2 cell line (HB-8065) and human normal liver cell line THLE-3 (CRL-11233) were prepared from American Type Culture Collection (ATCC) and were maintained in Dulbecco's modified Eagle's medium (DMEM), improved with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), and penicillin and streptomycin (100 µg/mL) and cultured at 37°C at 5% CO_2.

Cell viability assay

The cells (1×10^4 cells/ml) were incubated in a 12-well culture plate at 37°C and 5% CO_2 overnight, followed by the addition of RJ and 1 µM Doxorubicin, for 48 hr. In the next step, 50 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution (5 mg/mL, Sigma-Aldrich, St. Louis, MO) was added per tested well and then incubated for 4 hours at 37°C in darkness. Next, formazan crystals were dissolved by adding dimethyl sulfoxide (100 µ L) to each well. The absorbance of the tested plate at 570 nm was recorded by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).[16]

The morphological study

HepG2 cells were exposed with RJ (at the IC_{50} concentration) in 5% CO_2 at 37°C for 48 hours and then washed with phosphate-buffered saline. Cells are examined via inverted microscopy (Inverted Microsc°ope, Optika, Italy).

Annexin-V assay

To evaluate the initial and late apoptosis in HepG2 cells exposed with RJ, we used the Annexin-V (AV) assay using the dedicated kit (APOAlert® Annexin V; Clontech, Mountain View, CA, USA). At first, the HepG2 cells (1×10^5 cells/mL) were seeded at culture plates, and then, RJ at the ½ IC_{50} and IC_{50} concentrations was added to the cells for 24 hr. After washing the cells, they marked with AV-FITC and PI in the next step. A flow cytometer (BD Biosciences, San Jose, CA, USA) was utilized to analyze flow cytometry and the determination of apoptotic and necrotic cells.

Evaluating the gene expression by Real-time PCR

The gene expression of miRNA-34a (miR-34a), Bax, caspase-3, and Bcl-2 were assessed by real-time polymerase chain reaction (PCR). At first, total RNA of miRNAs was obtained from HepG2 cells by a Trizol reagent (Invitrogen, USA). A mirVana™ PARIS™ Kit (Qiagen, Germantown, MD, USA) was used to evaluate the expression of miR-34a. Initially, a TaqMan MicroRNA reverse transcription kit was applied to make the cDNA. Next, the SYBR green master mix was used to assess the expression of mature miR-34a, whereas a small nuclear RNA U6 gene was considered for standardization. To determine the gene expression of Bax, caspase-3, and Bcl-2 expression, the commercial kit of

Table 1: Sequences of primers used for real-time PCR

Primer	Sequence
Bax	5×-GGC TGG ACA CTG GAC TTC CTA-3×5×-GGTGAGGA CTCCAGCCACAA-3×
Caspase-3	5×-TGT TTG TGT GCT TCT GAG CC-3×5×-CAC GCC ATG TCA TCA TCA AC-3×
Bcl-2	5×- CAT GCC AAG AGG GAA ACA CCA GAA-3×5×- GTG CTT TGC ATT CTT GGA TGA GGG -3×
β-actin	5×- GTGACGTTGACATCCGTAAAGA-3×5×-GCCGGACTCATCGTACTCC-3×
miR-34a	5×-TGG CAG TGT CTT AGC TGG TTG T-3×5×-CGC TTC GGC AGC ACA TAT ACT AA-3×
U6 snRNA	5×-CGC TTC GGC AGC ACA TAT ACT AA-3×5×-TAT GGA ACG CTT CAC GAA TTT GC-3×

Qiagen, (Germantown, MD, USA) was applied to cDNA synthesis. The primer sequences used in real-time PCR are exhibited in Table 1. The reaction conditions were 96°C for 9 min, 40 cycles of 96°C for 10 s, and 57°C for 30 s. The fold change of mRNA expression levels in cells was measured by calculating the $2^{-\Delta Ct}$.

Western blot

At first, cell lysates were obtained, followed by exposure of HepG2 cells (1×10^4) with °RJ (1/4 IC_{50}, 1/3 IC_{50}, 1/2 IC_{50}, and IC_{50}) for 24 hr. After harvesting cells, cells were lysed in a protein lysis buffer. The protein was isolated by centrifuging the obtained cell lysates at 10000 g for 15 min, and its concentration was measured by Bradford assay. Then, proteins (20 µg/lane) were decomposed by 10% SDS-PAGE and transferred to a PVDF membrane (16). Next, by using the 5% skimmed milk, the prepared membranes were blocked in 0.1% TBST at 21°C and exposed with monoclonal primary antibodies for PARP (ab32064, Abcam, USA), Caspase-3 (ab32351, Abcam, USA), Caspase-9 (ab32539, Abcam, USA), Bcl-2 (ab59348, Abcam, USA), Bax (ab32503, Abcam, USA), and glyceraldehyde 3-phosphate dehydrogenase (GADPH) (ab8245, Abcam, USA) as controls. Then, they were exposed to HRP-conjugated secondary antibodies for 2 hrs at 21°C. Last, after washing the membranes in the buffer, the blots were visualized through chemiluminescence detection using an Amersham detection kit based on the manufacturer's guidelines and exposed to X-ray films. The bands related to protein expression level were scanned and measured by densitometric analysis.

Statistical analysis

For maximum validity, all trials were accomplished in triplicate. SPSS software version, 22.0 (SPSS Inc., Chicago, IL, USA), was used for analysis. The differences were studied by one-way ANOVA with Tukey's *post hoc* test.

RESULTS

Secondary metabolite analysis

The findings presented that the total phenolic, flavonoid, and protein contents were 76.3 ± 0.42 (mg GEA/g DW) and 2.23 ± 0.046 (mg QE/g DW) 14.4%, respectively.

Evaluation moisture and ash contents

The obtained results showed that moisture in the RJ sample was 60.2 ± 0.23%, whereas the ash content in the RJ sample was 0.13 ± 0.011%.

Cell viability assessment

Figure 1 shows the cell viability assessment of HepG2 cells treated with various concentrations of RJ (0.25, 0.5, 1, and 2 mg/ml), 1 µM doxorubicin, and RJ (at the concentration of ½ IC_{50}) plus 1 µM DOX as a combined treatment for 48 hr. The results demonstrated that viability of HepG2 cells after treatment with RJ was significantly ($P < 0.05$) reduced as a dose-dependent response. The IC_{50} value of RJ was found to be 1.13 mg/mL for HepG2 cells. RJ revealed no cytotoxicity on normal THLE-3 cells with IC_{50} >2 mg/ml. The results also exhibited that 1 µM DOX reduced the viability of HepG2 and normal THLE-3 cells by 42.6% and 73.6%, respectively.

Morphological study

A spindle specified the morphology of untreated cells with a nucleus and the same size. After incubation of HepG2 with RJ (at the concentration of ½ IC_{50}) for 48hr, cells displayed some morphological changes such as shorter protrusions, round shapes, smaller sizes, and cytoplasmic contraction [Figure 2].

Annexin-V assay

The early and late apoptosis in HepG2 cells treated with RJ (at the concentration of ½ IC_{50}) was evaluated by AV assay. As depicted in Figure 3, RJ at the concentration of ½ IC_{50} significantly increased ($p < 0.05$) apoptotic and necrotic cells from 0.96% to 28.3% and 9.3%, respectively. RJ at the concentration of IC_{50} significantly increased ($p < 0.05$) apoptotic and necrotic cells from 0.96% to 39.2% and 14.12%, respectively.

Evaluating the apoptosis-regulatory gene expression

The Bax and caspase-3 gene expression was considerably ($p < 0.001$) increased, ranging from 1.92 to 3.34-fold after treatment with RJ. The expression level of the miR-34a gene was noticeably ($p < 0.05$) elevated 2.11 to 4.45-fold after treatment with RJ. In contrast, the Bcl-2 expression level was considerably ($p < 0.05$) declined in the HepG2 cells exposed with RJ [Figure 4].

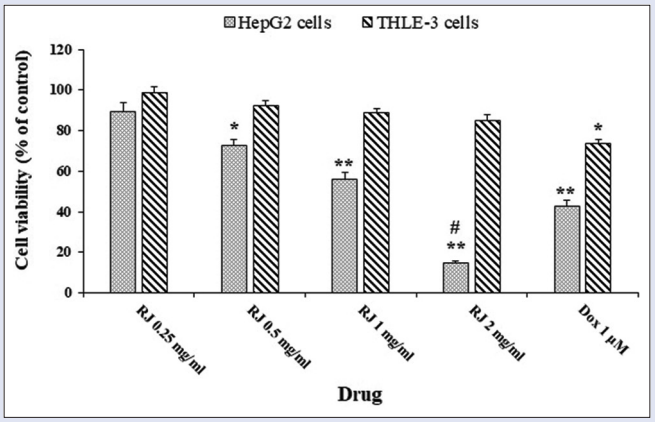

Figure 1: The cell viability assessment of HepG2 and THLE-3 cells treated with various concentrations of RJ (0.25, 0.5, 1, and 2 mg/ml) and 1 µM doxorubicin for 48 hr. * $P < 0.05$ difference was significant in comparison with the control; ** $P < 0.001$ difference was significant in comparison with the control; # $P < 0.001$ difference was significant in comparison with the control 1 µM doxorubicin (n = 3)

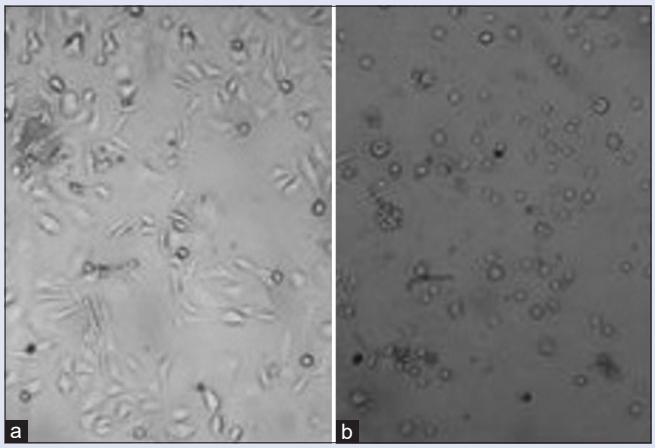

Figure 2: The morphology of untreated (a) and treated (b) HepG2 cells with RJ at the concentration of IC_{50} for 48 hr. Cells displayed some morphological changes such as shorter protrusions, round shapes, smaller sizes, and cytoplasmic contraction

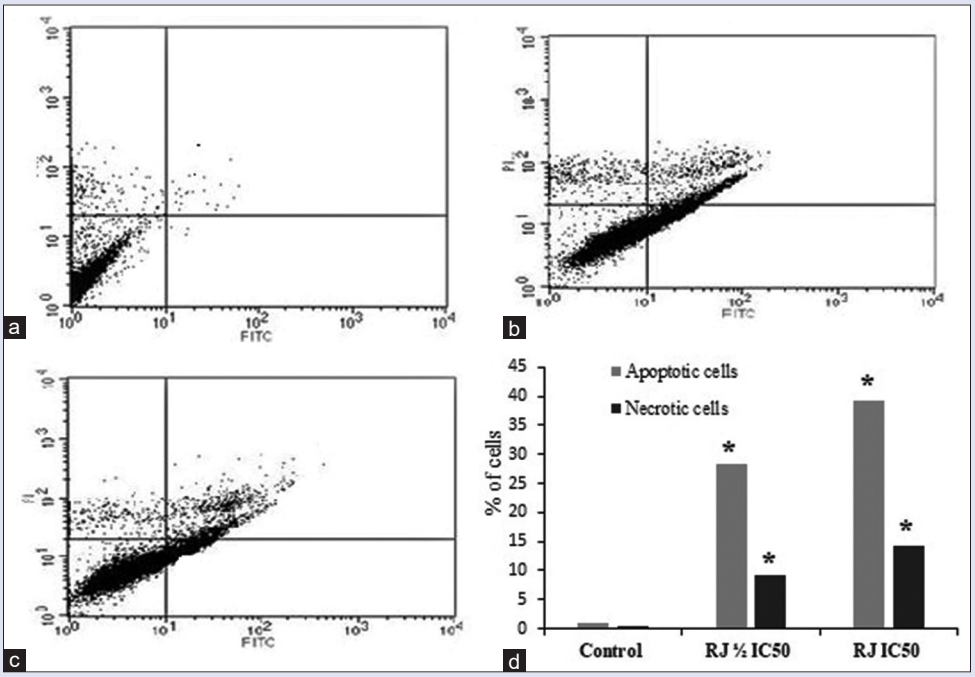

Figure 3: The early and late apoptosis in HepG2 cells treated with RJ at the concentrations of ½ IC_{50} and IC_{50} evaluated by AV assay. Control (a); RJ at the concentration of ½ IC_{50} (b) and RJ at the concentration of IC_{50} (c). percentage of apoptosis and necrotic in response to RJ at the concentration of ½ IC50 and RJ and RJ at the concentration of IC50 (d). Data are expressed as the mean ± SD (n = 3). * $P < 0.001$ difference was significant in comparison with the control

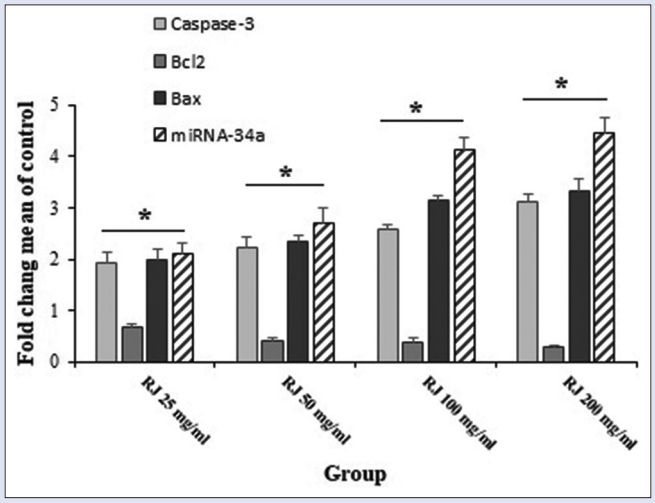

Figure 4: The expression level of the Caspase-3, Bcl-2, and Bax and miR-34a genes in HepG2 cells treated with various concentrations of RJ (0.25, 0.5, 1, and 2 mg/ml). Data are expressed as the mean ± SD (n = 3). * $P < 0.001$ difference was significant

Western blot

As shown in Figure 5, the Bcl-2 protein expression was predominantly inhibited after treatment of HepG2 cells treated with RJ (0.25-2 mg/ml), whereas treatment of HepG2 cells treated with RJ (0.25, 0.5, 1, and 2 mg/ml) resulted in a significant elevation in the protein expression of PARP, Caspase-3, Caspase-9, and Bax.

DISCUSSION

Nowadays, there are numerous synthetic drugs such as anti-metabolite agents for treating HCC; however, recent studies have reported various restrictions and side effects in the use of the synthetic agents such as bone marrow suppression, fatigue, nausea, vomiting, hair loss, and so on, as well as the expressions of multi-drug resistance genes.[10] RJ as a beneficial nutritious substance displayed numerous pharmacological characteristics such as anti-inflammatory, anti-oxidant, anti-cancer, neurotrophic, anti-diabetic, anti-microbial, and so on.[11] Here, we assessed the anti-cancer activity of RJ and its cellular mechanisms against the human hepatoma cell line HepG2.

Our results demonstrated that RJ (with the IC_{50} value of 1.13 mg/mL) mainly along with DOX meaningfully ($P < 0.05$) reduced the viability of HepG2 cells as a dose-dependent response so that the combination of RJ (at the concentration of ½ IC_{50}) along with plus 1 μM DOX showed that the highest cytotoxicity ($P < 0.001$) reduced the cell viability of HepG2 cells by 2.4%. Mohammadi *et al.*[17] have exhibited that RJ at the concentrations of 50 and 100 mg/mL exhibited a high cytotoxic effect on the prostate cancer cell line. In the study conducted by Nakaya *et al.*,[18] the results demonstrated that RJ has anti-cancer effects by suppressing the estradiol-induced cell proliferation of MCF-7 breast cancer cells. Miyata *et al.*[19] (2020) in a clinical trial study reported that oral administration of capsules having 900 mg of RJ considerably reduces the tumor size and some adverse side effects such as fatigue and anorexia in patients with renal carcinoma. In addition, Zhang *et al.*[20] (2017) have demonstrated that RJ at the doses of 0.5 and 1.5 g/kg meaningfully decreased the tumor weight in the 4T1 (breast tumor)-suffering mice.

Here, we found a considerable amount of the secondary metabolites such as total phenolic (76.3 mg GEA/g DW), flavonoids (1.23 ± 0.046 mg QE/g DW), and total protein content (14.4%) in RJ. Previous reports showed that RJ contains several bioactive compounds such as peptides, proteins, fatty acids (e.g., 10-hydroxydecanoic acid), polyphenols, and flavonoids (e.g., pinocembrin, quercetin, galangin).[21] Considering the anti-cancer effects of these compounds, Bhosale *et al.*[22] (2020) have demonstrated that the polyphenol compounds display their anti-cancer activity through several modes of actions such as elimination of cells via

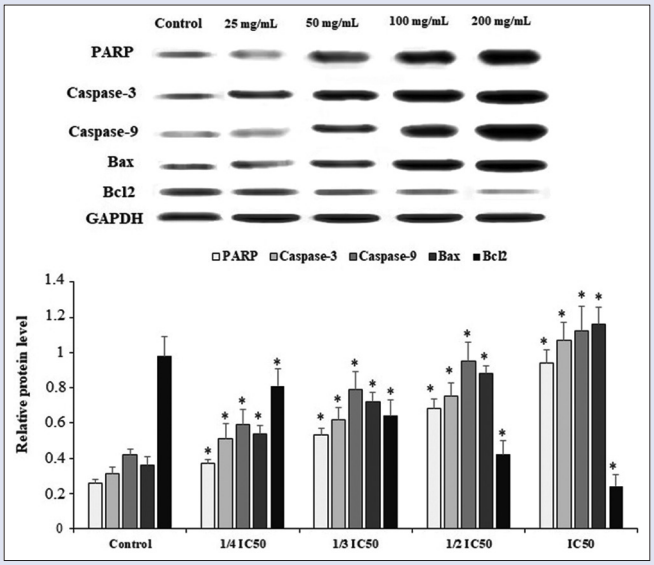

Figure 5: Effects of various concentrations of RJ (0.25, 0.5, 1, and 2 mg/ml) on the protein level of PARP, Caspase-3, Caspase-9, Bcl-2, and Bax in HepG2 cells. Data are expressed as the mean ± SD (n = 3). * $P < 0.001$ difference was significant compared with the control

signaling pathway alteration, suppression of cell cycle actions, apoptosis stimulation, and their anti-metastasis and anti-angiogenic properties. Moreover, Kopustinskiene *et al.*[23] (2020) have revealed that flavonoids exhibit their anti-cancer effects through mechanisms such as autophagy, controlling ROS-scavenging enzyme activities, suppressing the cell cycle, promoting the apoptosis, and inhibition of multiplication of cancer cells.

The incidence and development of cancer are often accompanied with abnormal proliferation and apoptosis resistance of cancer cells. Apoptosis is a gene-regulated process associated to extra-ordinary morphological changes, condensation of chromatin, and DNA damages.[24] Some factors and proteins are related to the mechanism of apoptosis, such as cysteine proteases (also called caspase enzymes and the Bcl-2 family). Among the caspases, Caspase 3 and 9 enzymes play a key role in the apoptotic process and are considered responsible for some mechanisms of apoptosis that cause DNA fragmentation, chromatin condensation, the cleavage of nuclear and cytosolic substrates and apoptotic bodies, and so on.[24,25] Instead, Bcl-2 as an anti-apoptotic prevents the apoptosis process through blocking cytochrome *c* release from mitochondria.[26] miR-34a is recognized as a tumor suppressor biomarker that has been used to assess and modulate cancer cell invasion, drug resistance, metastasis, diagnosis, and prognosis of cancers.[27] miRNA-34a shows different expressions in many cancer types such as colon cancer. Subsequently, down-regulation of miR-34a can disturb various processes such as the cell cycle, apoptosis, and differentiation and growth.[28,29] By real-time PCR, the expression of Bax and the Caspase-3 gene was considerably ($p < 0.001$) increased, ranging from 1.92 to 3.34-fold after treatment with RJ. The expression level of the miR-34a gene was noticeably ($p < 0.05$) elevated 2.11 to 4.45-fold after treatment with RJ. In contrast, the Bcl-2 expression level was considerably ($p < 0.05$) declined in the HepG2 cells exposed with RJ.

By AV assay, HepG2 cell treatment with RJ at the concentration of ½ IC_{50} significantly increased ($p < 0.05$) apoptotic and necrotic cells from 0.96% to 28.3% and 9.3%, respectively. RJ at the concentration of IC_{50} significantly increased ($p < 0.05$) apoptotic and necrotic cells from 0.96% to 39.2% and 14.12%, respectively.

PARP is considered an important enzyme involved in the synthesis of the chromatin structure, replication, transcription, and DNA restoration.[30] Previous studies revealed that up-regulation and hyper-activation of PARP result in cell death through a specific apoptosis pathway described by mitochondrial dysfunction, depletion of NAD+/ATP, imbalance of calcium, and release of the apoptosis-inducing factor.[30,31] Our results exhibited that HepG2 cells treated with various concentrations of RJ (0.25, 0.5, 1, and 2 mg/ml) increased PARP expression, indicating that RJ might induce cell death through a specific apoptosis pathway.

CONCLUSION

Our results showed the promising anti-cancer effects of RJ against HepG2 cells, whereas the induction of apoptosis by various pathways is considered as the main mechanism underlying the cytotoxic effect of RJ against HepG2 cells. The present study's findings propose that RJ can be a candidate agent for treating human HCC.

Acknowledgements

The authors extend their appreciation to the Deputyship for Research & innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number IFP2021-047.

Financial support and sponsorship

This research was funded by the Deputyship for Research & innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number IFP2021-047.

Conflicts of interest

There are no conflicts of interest.

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