Investigating the effect of rubiadin cytotoxicity and expression of BAX and BCL2 genes on HepG2 liver cancer cell line

Sanaz Pashapour1, Abbas Zabibi2✉, Yeganeh Hamidi3, Masoumeh Heshmati4

1Department of Pharmacology and Toxicology, Faculty of Pharmacy and Pharmaceutical Sciences, Tehran Medical Sciences, Islamic Azad University, Tehran 009821, Iran. 2Department of Biology, Faculty of Basic Sciences, Islamic Azad University, Rasht Branch, Rasht 0098813, Iran. 3Department of Biology, Faculty of Basic Sciences, East Tehran Branch, Islamic Azad University, Tehran 009821, Iran. 4Department of Cellular and Molecular Biology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran 009821, Iran.

*Corresponding to: Abbas Zabibi, Department of Biology, Faculty of Basic Sciences, Islamic Azad University, Rasht Branch, Ibn Sina St, Rasht District 4, Iran Rasht 0098813, Iran. E-mail: zabibi.abbasz@gmail.com.

Author contributions
S. P. and A. Z. conceived the study. S. P. performed data analysis and interpretation and drafted the manuscript. Y. H. critically revised the text for main intellectual content, and A. Z. performed the statistical analysis.

Competing interests
The authors declare no conflicts of interest.

Acknowledgments
The present research received financial support from the Pharmaceutical Sciences Branch of Islamic Azad University.

Peer review information
Precision Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations
MTT, 3-(4,5)-dimethylthiahiazo [-z-y1]-3,5-di-phenyterazoliumromide.

Citation

Executive editor: Xun-Yun Zhang.

Received: 07 June 2023; Accepted: 14 July 2023; Available online: 28 July 2023.
© 2023 By Author(s). Published by TMR Publishing Group Limited. This is an open access article under the CC-BY license.

Abstract
Background: Rubiadin is a type of anthraquinone compound that can be found in Rubiaceae plants, such as Ronas. Nonetheless, only limited research has been done to explore the potential anticancer properties of rubiadin on liver cancer cells. Thus, the objective of the present study is to examine how rubiadin affects the viability of liver cancer cells as well as normal cells. Methods: HepG2 and AGO cell lines were assigned into controls (not exposed to rubiadin) and groups with exposure to rubiadin with 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 μg/mL concentrations. 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenyterazoliumromide and reverse transcription-polymerase chain reaction were used to measure cell viability, and one-way analysis of variance was used for data analysis. Results: The viability of liver cancer cells was significantly reduced when exposed to 12.5, 6.25, 3.125, and 1.56 μg/mL concentrations (P < 0.01). An IC50 of 44.73 μg/mL was reported. Furthermore, the BAX gene’s relative expression (P < 0.05) was significantly increased and the BCL2 gene expression (P < 0.05) was significantly reduced. The average ratio of BAX gene expression to BCL2 increased significantly (P < 0.01). Conclusion: This research showed that rubiadin decreases cell viability by increasing the ratio of BAX gene expression to BCL2. In addition rubiadin has no cytotoxic effect on normal cells.

Keywords: rubiadin; viability; AGO; HepG2; BAX; Bcl2; BAX/BCL2
Introduction

Liver cancer is the third death factor in the world, including Iran [1]. The International Agency for Research on Cancer reported over eight hundred thousand deaths from liver cancer in 2020 [2]. Currently, surgery is the most successful approach for treating liver cancer, and other approaches have lower effectiveness [3-5]. Many studies have reported that the Morinda citrifolia plant was identified as a factor in the prevention or treatment of cancers [6]. The Noni plant or Morinda citrifolia (Indian mulberry) and the rubiadin family produce a substance called rubiadin [7]. The root of this plant can be used in the treatment of all kinds of cancers. Due to the lack of investigation of the anticancer impact of this pure substance up to now, and due to the observation of cytotoxic impacts from plants containing rubiadin, we decided to use it for the first time and examine its anti-cancer effects. Studies on Morinda citrifolia show that this substance may induce cytotoxic impacts in cancer cells [7]. Reports indicate that rubiadin can have a function in inducing the gene expression in cancer cells [8, 9]. The Bcl2 gene is engaged in regulating cell apoptosis process and is an anti-apoptotic gene. If its expression is reduced, the process of programmed cell death starts [10]. On the other hand, the BAX protein or Bcl2-dependent protein s is an apoptosis regulatory protein encoded by the BAX gene, which regulates apoptosis. Increasing the ratio of the BAX gene to the Bcl2 gene has a key function in inducing apoptosis [11]. Given the extent and prevalence of liver cancers worldwide, including Iran [2, 12], and the other findings regarding the effects of it on plant all types of cancer cells [7, 13], the present study aims to analyze and research rubiadin cytotoxic effects on liver cancer cells. The present study, which provide new outcomes, offers essential data for clinical and research purpose. Since studies on the anticancer impacts of rubiadin in liver cancer cells have been scarce, this research investigated the rubiadin cytotoxicity effects on HepG2 liver cancer cells.

Materials and methods

The special cell line linked to normal cells (AGO) and liver cancer (HepG2) was acquired at the Institut Pasteur (Paris, France). To determine the levels, 0.5 μL of dimethyl sulfoxide solution was added to 10 grams of rubiadin. Subsequently, it was prepared by diluting the required concentrations. As part of this research, rubiadin was prepared as a 10 mg pure powder from Shahar Azma Co., (Tehran, Iran) at all concentrations has been prepared. During this study, liver cancer cell line (HepG2) and normal (HGF) was randomly assigned to the control group (not exposed to rubiadin) and the groups affected by rubiadin with concentrations of 12.5 and 6.25, 3.125, 1.56, 0.78 and 0.39 μg/mL were divided. Survival rates were measured using the 3-(4,5-dimethylthiazol-2-yl)-3,5-di-(phenyl)-tetrazolium bromide (MTT) method and the epoch-BioTek (enzyme-linked immunosorbent assay) drive.

MTT assay

In this respect, to measure MTT, 1 × 10^5 HepG2 and AGO cancer cells were grown in each well with Dulbecco’s Modified Eagle Medium (Sigma-Aldrich Corp., St. Louis, MO, USA) culture medium. The levels of rubiadin in the cells were 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 μg/mL after 24 hour. In addition, 72 hours of exposure to was 20 μL of MTT solution (Sigma-Aldrich Corp., St. Louis, MO, USA) were added. Finally, following incubating the plates and thoroughly draining the growing medium, we added dimethyl sulfoxide solution from Yekta Tajhiz Azma Co., Company (Tehran, Iran) (200 μL) to the wells [14, 15].

Measurement of BAX and Bcl2 genes

HepG2 cancer cells were treated for 72 hours with the selected concentrations of the rubiadin.

Extraction RNAs

Total RNA was extracted in accordance with the manufacturer’s literature (cat: YT9080) and then synthesized cDNA (cat: YT4500) from Yekta Tajhiz Azma Co., (Tehran, Iran).

Synthesis cDNA

As an initial step, we added 1 μL of primer to 1 μL of the RNA sample. The desired solution has been increased to 13.4 with dialyzed pyrocarnate water (Yekta Tajhiz Azma Co., Tehran, Iran). After this, the solution was incubated for 5 minutes at 70 °C. Eventually, the standard buffer, d NTP, RNasin and MLV (Yekta Tajhiz Azma Co., Tehran, Iran) were added to the super standard solution. The microtube was incubated by polymerase chain reaction for 60 minutes at 42 degrees celsius. The level of gene expression was determined by Amplicon master mix kit and reverse transcription-polymerase chain reaction with a Step One ABI machine. The Livak 2−ΔΔCT method was used to analyze the gene expression data.

Statistical analysis

The results were statistically analyzed by GraphPad Prism software version 8.1.0. Analysis of variance and Tukey’s range test were employed for the analysis, with P-value of ≤ 0.05 as statistically significant.

Results

The viability rate of liver cancer cells, it was found that there was a significant reduction in viability rate of HepG2 liver cancer cells when exposed to 12.5, 6.25, 3.125, and 1.56 μg/mL concentrations compared to control group (P < 0.01). 0.78 μg/mL and 0.39 μg/mL concentrations did not show any significant differences. The half-maximal inhibitory concentration (IC50) was 44.73 μg/mL, as illustrated in Figure 1.

Figure 1 The viability of HepG2 cells. The sign signifies the comparison of investigated and control groups. *P < 0.01.
Investigating the viability rate in normal AGO
The study found that no significant differences were noted compared to the control group in viability rate of normal cancer cells treated with AGO at 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 μg/ml concentrations (Figure 2).

Evaluation of gene expression in HepG2 cell lines
The IC₅₀ for liver cancerous cells was 44.73 μg/ml. The results showed a notable rise in the BAX gene's relative expression compared to the control (2-fold), as well as a decrease in the BCL2 gene's relative expression (2.5-fold) compared to the control, with a statistical significance of P < 0.05. Furthermore, significant differences with a P < 0.01 were observed in the average ratio of BAX/BCL2 gene expression, which was found to be 15-fold higher than the control (as shown in Figure 3).

Discussion
Many researchers have determined the ability of the rubiadin in decreasing the activity of cancer cells [8, 16], but the positive effects of rubiadin on decreasing the cancer cells' division, particularly liver cancer, is important research subjects. Based on this study examined the rubiadin effects on HepG2 liver cancer cells by using MTT method and reverse transcription-polymerase chain reaction, to show that rubiadin can prevent the growth of liver cancer cells before the advanced stage. Findings from this research show that levels of 12.5, 6.25, 3.125 and 1.56 μg/mL induce cytotoxic impacts in liver cancer cells. Moreover, by increasing the concentration of rubiadin, the cytotoxic impact on liver cancer cells increases. Gene expression results also demonstrated that an IC₅₀ of 44.73 μg/mL rubiadin increases BAX/BCL2 in liver cancer cells. Consistent with these conclusions, Zabihi, et al. studied the cytotoxic effect of rubiadin of MCF7 cell line and reported that this substance elevates the BAX/BCL2 expression and decreases the mammary cell line viability [8]. In one study, Dr. Kamiya investigated the cytotoxic effect of rubiadin present in Morinda citrifolia and observed that rubiadin reduced cancer cell survival [16]. Morinda citrifolia was found to have antinecancer properties. The cytotoxic effect of rubiadin on liver cancerous cells is possible, according to earlier studies [8]. In one study, Dr. Nasarudin Watroly reported the cytotoxic impact of rubiadin in cervical, mammary and leukaemia cancer cells [17].
study demonstrated the cytotoxic impact of Morinda citrifolia anthraquinone on colon cancer cells [18]. Rubiadin likely has an effect through the cell membrane [17]. Further studies are required for exploring the rubiadin impact at different concentrations on the expression of molecules that regulate the process of apoptosis, which can provide knowledge regarding the mechanism of action of rubiadin in inducing cell death in liver cancer cells.

**Conclusion**

Rubiadin was shown to cause cell viability reduction in liver cancer cells. The IC₅₀ for liver cancerous cells was 44.73 µg/mL. A 2-fold increase in the BAX gene expression and a decline 2.5 -fold in the relative BCL2 gene expression have been reported. Moreover, the mean expression ratio of the BAX gene to BCL2 (15 times) was observed. Rubiadin does not have cytotoxic impact on normal cells.

**References**


