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Syringic acid induces cancer cell death in the presence of Cu (Ⅱ) ions *via* pro-oxidant activity

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ABSTRACT

Objective: To investigate the effects of syringic acid on HEK 293 and HepG2 cells in the absence and presence of exogenous Cu (Ⅱ) and Fe (Ⅱ) ions.

Methods: The antiproliferative effects of syringic acid on HEK 293 and HepG2 cells in the absence and presence of exogenous Cu (Ⅱ) and Fe (Ⅱ) ions were examined by MTT assay. Additionally, colony-forming, reactive oxidative species (ROS) generation, apoptosis induction, autophagy, mitochondrial membrane potential, and mitochondrial mass were investigated.

Results: At 24 and 72 h, no significant differences were observed in the viability of HepG2 cells between the control and syringic acid + Fe (Ⅱ) groups. However, exposure of HepG2 cells to syringic acid + Cu (Ⅱ) for 72 h reduced the cell viability significantly. Furthermore, ROS formation, induction of apoptosis, and autophagic vacuoles were significantly increased in HepG2 cells without marked changes in mitochondrial membrane potential and mitochondrial mass. Moreover, syringic acid + Cu (Ⅱ) reduced the plating efficiency and surviving fraction significantly.

Conclusions: The combination of syringic acid with Cu (Ⅱ) was toxic to cancer cells and showed pro-oxidant activity. In addition, this combination induced autophagy in cancer cells with less cytotoxic effects on normal cells, which is a potential candidate for the development of novel therapeutics towards cancer.

KEYWORDS: Syringic acid; Copper; Iron; Cancer cell; Pro-oxidant; Autophagy

1. Introduction

Recently, oxidation therapy or use of pro-oxidants effects of antioxidants is a novel strategy in cancer treatment. Antitumor activities of several available drugs are related to reactive oxygen species (ROS) formation. Paclitaxel, cisplatin, doxorubicin, arsenic trioxide, and etoposide can form H_2O_2 by NADPH oxidase activation[1]. It seems that cancer cells are more susceptible to H_2O_2 in comparison to normal cells. For instance, the high dose of ascorbic acid inhibited the tumor progression by generating H_2O_2 without toxicity effects on normal tissues[2]. Mechanisms of selective toxicity of pro-oxidants to cancer cells may be related

Significance

Syringic acid, a natural phenolic acid, showed significant cytotoxicity in HepG2 in the presence of Cu (Ⅱ). The combination of syringic acid and Cu (Ⅱ) induced apoptosis and increased cell population in sub-G_1 and autophagic vacuoles *via* ROS formation. Syringic acid combined with Cu (Ⅱ) may be useful in cancer treatment.

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to the ability of these cells to produce higher concentrations of H_2O_2 than normal cells[3]. Also, some tumor cells contain more amounts of copper and iron metals which can generate ROS through Fenton reaction[4,5]. Phenolic compounds are known as secondary metabolites of plants with a wide range of therapeutic effects. They possess both antioxidant and pro-oxidant effects in different concentrations and cellular conditions[1,6,7]. Great attention has been paid to applying antioxidants in treatment of different diseases such as neurodegenerative disease[6,8–10], diabetes[11–13], inflammation, and modulation of side effects of chemotherapeutic protocols in cancer treatment[14].

The presence of transition metal ions is involved in oxidation processes. Also, high pH and high concentration of phenolic compounds can prepare the condition to reveal the pro-oxidant activity of these compounds[6]. Direct ROS production is one of their mechanisms to alter the redox cellular environment. Moreover, some phenolic compounds such as caffeic acid and ferulic acid can induce the intracellular production of ROS and induce apoptosis by NADPH oxidase pathway[15]. Furthermore, phenolic compounds reduce metal ions and stimulate the formation of hydroxyl radicals through Fenton reaction[1].

Syringic acid (SYR) or *O*-methylated trihydroxy benzoic acid is one of the natural phenolic compounds, which exhibits antioxidant, anti-inflammatory, anti-diabetic, and hepatoprotective effects in various animal models; in addition, it showed a cytotoxic effect in several cancer cell lines[16].

Drug resistance to different chemotherapeutic agents raises serious challenges in cancer treatment. As the global incidence of cancers increases, improving chemotherapy efficacy is more considerable[17]. Much effort has been devoted to developing a new mechanism to improve the efficacy of cancer chemotherapy[18].

Recent data have shown that a number of genetic and epigenetic mechanisms lead to resistance of cancer cells to apoptotic cell death[19]. Thus, it is suggested that some compounds that induce cell death through autophagy could be new therapeutic agents in cancer treatment. Previous studies revealed that in our diet, natural polyphenolic compounds including genistein, rottlerin, curcumin, quercetin, and resveratrol can induce cell death *via* autophagy[19]. Consequently, phenolic compounds are used as a co-treatment with standard therapies in cancer[19].

Autophagy is the cellular regulatory process that removes unnecessary or dysfunctional components. This pathway helps the cells to keep cellular homeostasis. It can be done by selective and non-selective mechanisms[20]. Mitophagy is the selective degradation of mitochondria through autophagy. This is an important cellular pathway to control mitochondrial quality. Thus, this process plays a critical role in normal cell development[21]. Oncogenic stresses lead to uncontrolled mitochondrial turnover and result in either stimulation or suppression of tumor genesis process[21]. Accordingly, the study of mitophagy, cell death, and tumorigenesis simultaneously can reveal the targets involved in the stimulation of cell death in cancer cells and discovery of new anticancer agents[21].

In this regard, the polyphenols could be considered potent autophagy modulators for cancer therapy. In this study, the pro-oxidative and antiproliferative effects of SYR on normal and cancer cells in the absence and presence of exogenous Cu and Fe ions were analyzed and compared. To monitor the mechanism of co-treatment of SYR and transition metals in cell death, we analyzed colony formation, ROS generation, and apoptosis induction. Also, mitochondrial membrane potential, mitochondrial mass, and autophagy were investigated.

2. Materials and methods

2.1. Reagents

SYR, $CuSO_4$, and $FeSO_4$ were purchased from Merck Company (Germany). 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine 123, propidium iodide (PI), and monodansylcadaverine (MDC) were obtained from Sigma (St. Louise, MO, U.S.A.). 10-*N*-nonyl acridine orange was purchased from Santa Cruz Biotechnology.

2.2. Cell culture and cytotoxicity assay

The HepG2 (human liver cancer cell) and HEK 293 (human embryonic kidney cells) were obtained from Pasteur Institute (Tehran, Iran). They were maintained at a 37℃ incubator under 5% CO_2 and cultured in RPMI 1640 supplement containing 10% fetal bovine serum and 1% penicillin-streptomycin. After reaching 80% confluency, cells were harvested by trypsin. Different concentrations of samples were added to the cells and cell viability was examined by MTT assay. Briefly, 2000 cells/well of HepG2 or HEK 293 cells were seeded in 96-well cell culture plates. After 24 h, growth media were replaced with a fresh growth medium containing different concentrations of SYR (50-1000 μM) or Fe (Ⅱ) (50-500 μM) or Cu (Ⅱ) (50-300 μM) and incubated for 24 and 72 h. At the end of treatment, the medium was replaced with MTT solutions (0.5 mg/mL) and was incubated at 37℃ for 4 h. The formazan obtained was solubilized with DMSO. The absorption was measured at 570 nm by using a microplate reader (BioTek instrument). After selecting the concentration of SYR, Fe (Ⅱ), and Cu (Ⅱ), we co-treated the cells with SYR or metal ions for 24 and 72 h to measure the cytotoxicity by MTT assay.

2.3. Flow cytometry analysis

PI staining was used for the cell cycle analysis and the percentage of cells in the sub-G_1 phase was evaluated for the number of apoptotic cells. The cells were grown in 6-well plates (5×10^5 cells/well) and treated with samples for 72 h. Then the cells were

harvested by trypsin and fixed in 70% ethanol by incubation on ice for 2 h. Subsequently, the cells were washed with phosphate buffer saline (PBS) and incubated in the dark with a mixed buffer of 50 μg/mL PI and 25 μg/mL RNase A for 30 min before flow cytometric analysis of the Fl2 channel[22].

2.4. Colony formation assay

The base of this assay is related to the ability of a single cell to grow into a colony. After treatment of the cells with samples, they were removed by trypsin and seeded in 6 well plates in a low density of about 500 cells and incubated for one week. Colonies were fixed with 100% methanol, stained with crystal violet (0.5% *w/v*), and counted using a stereomicroscope.

2.5. Intracellular ROS analysis

The cells were treated with suitable concentrations of the samples for 72 h and then incubated at 37℃ with DCFH-DA (10 μM) for 30 min in the dark. The cells were washed with phosphate buffer and ROS generation was measured using the fluorescence intensity by the microplate fluorimeter (BioTek instrument) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm[23].

2.6. Mitochondrial membrane potential

Mitochondrial potential (Δψmit) changes were evaluated by measuring rhodamine-123 fluorescence quenching under the following conditions. After treatment of the cells with samples, they were washed with PBS and incubated with 10 μM rhodamin at 37℃ for 30 min. The fluorescence intensity was analyzed by fluorimeter (excitation 485 nm and emission 520 nm wavelength)[24].

2.7. Labeling of autophagic vacuoles

MDC is known as a selective fluorescent dye used to observe autophagic vacuoles. Following treatment with samples, the cells were incubated with 10 μM MDC in PBS at 37℃ for 30 min. After incubation, the cells were washed with PBS and visualized by a fluorescence microscope (Olympus BX61 and BP470/BA510 filter) and analyzed by Image J software[25].

2.8. Mitochondrial mass analysis

After treatment with samples, cells were washed with PBS and incubated with 10-*N*-nonyl acridine orange (2.5 μM) at 37℃ for 30 min. After incubation, the cells were washed with PBS and collected to analyze the fluorescence intensity by a microplate fluorimeter at an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. The slides of cells were prepared and the images were obtained by a fluorescence microscope (Olympus BX61 and BP470/BA510 filter) and analyzed by Image J software[26].

2.9. Statistical analysis

Data are presented as mean ± SD. Data comparison was performed using a one-way analysis of variance with Dunnett post-test. SPSS 16.0 software was used for the statistical analysis. $P < 0.05$ was considered to be statistically significant.

2.10. Ethical statement

This study was approved by the Ethics Committee of the University of Shiraz University of Medical Sciences (Ethical Code: IR.SUMS.REC.1397.1083).

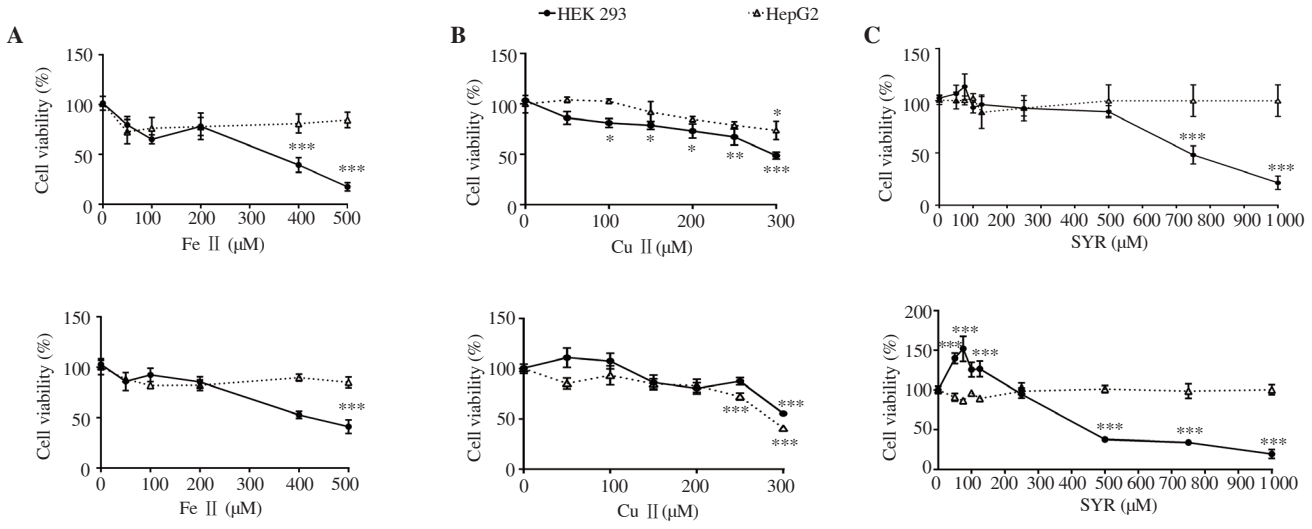

Figure 1. Effect of different concentrations of $FeSO_4$ (50-500 μM) (A), $CuSO_4$ (50-300 μM) (B), and SYR (50-1 000 μM) (C) on HEK 293 and HepG2 cell viability after 24 h (upper) and 72 h (lower) of incubation. Cell viability was determined using MTT assay. The data are expressed as mean ± SD of at least three independent experiments and analyzed by one-way analysis of variance followed by Dunnett post-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the control group. SYR: Syringic acid.

3. Results

3.1. Cytotoxicity assay

The results showed that Fe (Ⅱ) at 500 μM significantly reduced the viability of HEK 293 after 24 and 72 h (P<0.001) (Figure 1A). However, Fe (Ⅱ) at all tested concentrations did not show significant toxicity to HepG2 cells after 24 and 72 h. In contrast, the viability of HEK cells treated with 100-300 μM Cu (Ⅱ) and 300 μM Cu (Ⅱ) treated HepG2 cells was significantly decreased after 24 h of incubation (P<0.001). In addition, 250 and 300 μM of Cu (Ⅱ) caused significant toxicity to HepG2 cells after 72 h of incubation (P<0.001) (Figure 1B).

SYR at concentrations over 700 μM was toxic to HEK cells after 24 h of incubation (P<0.001), while it did not show any significant toxicity to HepG2 cells (Figure 1C). Moreover, SYR at 50-150 μM markedly increased the viability of HEK cells when compared to the control (P<0.001) after 72 h, but 500-1 000 μM SYR decreased the HEK cell viability significantly (P<0.001). However, SYR (0-1 000 μM) was not cytotoxic to HepG2 cells (Figure 1C).

The safe and non-cytotoxic concentrations of Fe (Ⅱ), Cu (Ⅱ), and SYR in the HEK cell were considered for co-treatment. The HepG2 cells were treated with SYR (125 and 250 μM) and Fe (Ⅱ) 100 μM for 24 and 72 h. After 24 and 72 h of incubation, there were no differences between the viability of the control and treated groups in the HepG2 cells (Figure 2A).

The HepG2 and HEK cells were incubated with different concentrations of SYR (125 and 250 μM) and Cu (Ⅱ) (100 and 250 μM) for 24 and 72 h. After 24 h incubation, 100 and 250 μM of Cu (Ⅱ) and all co-treatment of Cu (Ⅱ) and SYR reduced the viability of the HEK cells (P<0.05 and P<0.001). The combination of 100 μM Cu (Ⅱ) and 250 μM SYR showed a significant reduction in cell viability in comparison to 100 μM Cu (Ⅱ) alone. Moreover, a prominent decrease in HEK cell viability was observed after treatment with Cu (Ⅱ) 250 μM and SYR 250 μM. Furthermore, treatment of HEK cells with Cu (Ⅱ) and SYR in all concentrations did not show any significant toxicity after 72 h (Figure 2B). In contrast, the combination of Cu (Ⅱ) + SYR in all concentrations did not cause any changes in HepG2 cell viability after 24 h, but treatment with Cu (Ⅱ) (250 μM) and SYR (125 and 250 μM) significantly reduced the viability of HepG2 cells after 72 h (P<0.05) (Figure 2C).

3.2. Cell cycle and apoptosis

To explore the effect of the combination of SYR + Cu (Ⅱ) on cell cycle progression, we examined the cell cycle distribution of HepG2 cells by flow cytometry after 72 h exposure to this combination (Table 1 and Figure 3). In agreement with the aforementioned MTT results, Cu (Ⅱ) increased the proportion of cells in the sub-G_1 phase, with a significant induction in apoptosis (P<0.001). Likewise, the cells exposed to SYR+Cu (Ⅱ) showed an increased proportion of cells in the sub-G_1 and G_2/M phases in comparison to the cells that received only Cu (Ⅱ) (P<0.001 and P<0.05, respectively).

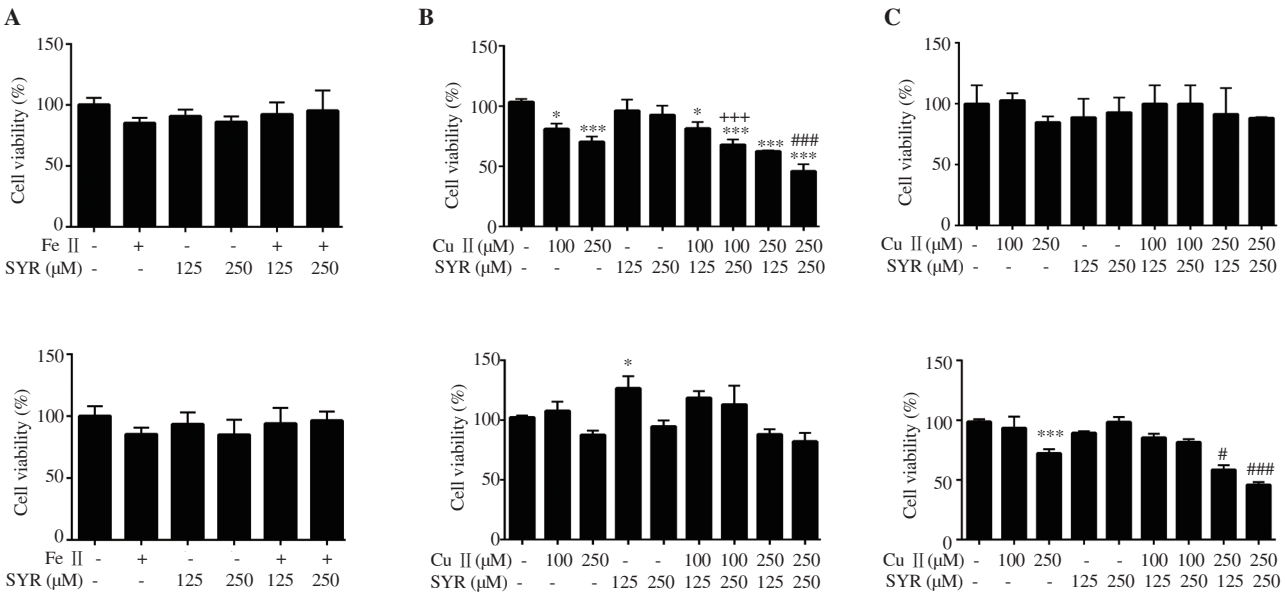

Figure 2. Effect of co-treatment with SYR + Fe (Ⅱ) or SYR + Cu (Ⅱ) on the cell viability of HEK 293 and HepG2 after 24 h (upper) and 72 h (lower) of incubation. (A) SYR (125 and 250 μM) + Fe (Ⅱ) 100 μM in HepG2 cells; (B and C) SYR (125 and 250 μM) + Cu (Ⅱ) (100 and 250 μM) in HEK 293 and HepG2 cells. Cell viability was determined using MTT assay. The data are expressed as mean ± SD of at least three independent experiments and analyzed by one-way analysis of variance followed by Dunnett post-test. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with the control group. $^{+++}P < 0.001$ compared with the Cu (Ⅱ) (100 μM) group. $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with the Cu (Ⅱ) (250 μM) group.

Table 1. Effect of co-treatment with SYR and Cu (Ⅱ) on DNA fragmentation in HepG2 cells (% cells).

Groups	Sub-G_1	G_2/M
Control	5.3±0.86	17.1±1.4
SYR 250 μM	4.7±1.90	18.2±2.2
Cu (Ⅱ) 250 μM	34.7±4.20***	11.5±1.5
SYR 250 μM+Cu 250 μM	46.4±4.50###	27.5±3.2#

The data are expressed as mean ± SD of at least three independent experiments and analyzed by one-way analysis of variance followed by Dunnett post-test. ***$P < 0.001$ compared with the control group. #$P < 0.05$, ###$P < 0.001$ compared with the Cu (Ⅱ) (250 μM).

3.3. Colony-forming assay

According to the results of colony-forming assay, after 72 h incubation, SYR (250 μM) increased the plating efficiency and a surviving fraction of the treated HepG2 cells in comparison to the controls (P<0.01). Moreover, Cu (Ⅱ) (250 μM) and SYR 250 μM+Cu 250 μM significantly reduced the plating efficiency and surviving fraction in comparison to the control and Cu 250 μM groups (P<0.001) (Table 2, Supplementary Figure).

3.4. Intracellular ROS analysis and mitochondrial membrane potential

ROS formation was significantly increased in the HepG2 cell exposed to 250 μM Cu (Ⅱ) (P<0.001). Also, ROS levels were significantly elevated in the cells treated with 250 μM SYR and 250 μM Cu (Ⅱ) in comparison with the cells treated with 250 μM Cu (Ⅱ) only after 72 h (P<0.001) (Figure 4A). No change in mitochondrial membrane potential was observed after treatment (Figure 4B).

Table 2. Effect of co-treatment with SYR and Cu (Ⅱ) on colony-forming in HepG2 cells.

Groups	Plating efficiency	Surviving fraction
Control	0.39±0.02	1.00
SYR 250 μM	0.48±0.01**	1.20**
Cu (Ⅱ) 250 μM	0.29±0.02**	0.74**
SYR 250 μM+Cu 250 μM	0.14±0.01***###	0.36***###

Plating efficiency: number of colonies formed/number of cells inoculated; Surviving fraction: (number of colonies formed/number of cells inoculated)/ plating efficiency control group. **$P < 0.01$, ***$P < 0.001$ compared with the control group. ###$P < 0.001$ compared with the Cu (Ⅱ) (250 μM).

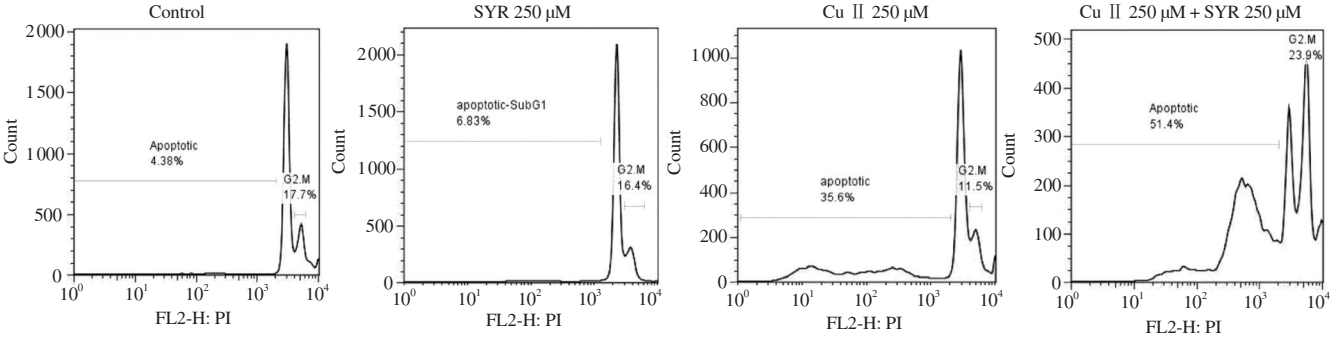

Figure 3. Effect of co-treatment with SYR and Cu (Ⅱ) on DNA fragmentation in HepG2 cells. DNA fragmentation was determined by flow cytometry analysis.

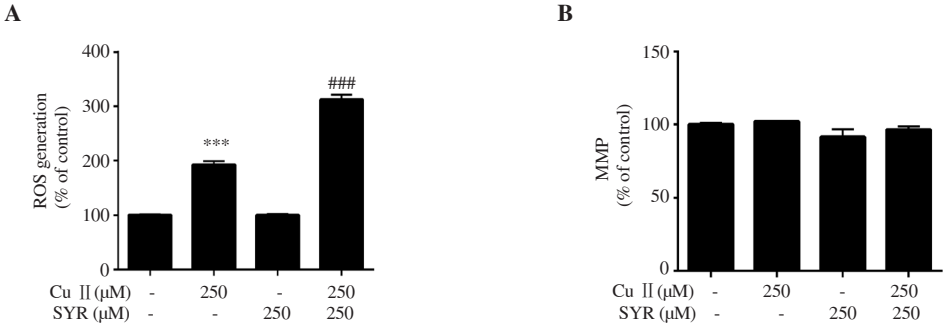

Figure 4. Effect of co-treatment with SYR and Cu (Ⅱ) on ROS generation (A) and mitochondrial membrane potential (MMP) (B) in HepG2 cells. ROS formation was determined by DCFH-DA and MMP was determined by rhodamine 123. The data are expressed as mean ± SD of at least three independent experiments and analyzed by one-way analysis of variance followed by Dunnett post-test. ***$P < 0.001$ compared with the control group. ###$P < 0.001$ compared with the Cu (Ⅱ) group (250 μM).

3.5. Visualization of MDC–labeled vacuoles and mitochondrial mass

As shown in Figure 5A, co-treatment of SYR+ Cu (Ⅱ) increased the MDC-labeled vacuoles in comparison to the Cu (Ⅱ) group after 72 h. There was no statistically significant difference in the mitochondrial mass following treatment with Cu (Ⅱ) and SYR+ Cu (Ⅱ) (Figure 5B).

4. Discussion

Differences between normal and cancer cells are a critical issue in developing novel cancer treatment. Targeting particular processes that are more vulnerable in cancer cells would reduce the side effects of therapeutics. It is supposed that pro-oxidant compounds can be tolerated by normal cells, but they can induce cell death in cancer cells under oxidative stress conditions[27]. Therefore, in this study, the possible pro-oxidant effects of SYR in the presence of Cu (Ⅱ) and Fe (Ⅱ) in cancer and normal cells were investigated.

Imbalance of redox-active metal homeostases like Fe and Cu leads to formation of extra ROS and RNS levels in the cell. Furthermore, redox-active metals can bind to phospholipids, disturb the integrity of lipid bilayers, and make them prone to lipid peroxidation[28]. Also, iron deregulation can play a critical role in cancer risk, metastasis, survival, and iron-mediated metabolism[29].

In our study, 500 μM of Fe (Ⅱ) reduced the viability of HEK cells significantly after 24 and 72 h. However, this concentration did not show any significant effects on the HepG2 cell viability. It seems that Fe (Ⅱ) is more toxic to normal cells in comparison to cancer cells. It can refer to the ability of HepG2 cells to increase ferritin expression when exposed to iron. Yet, in normal cells, the pro-oxidant activities of iron may lead to damage to protein synthesis pathways involved in iron deposits. Consequently, these cells are more sensitive to high concentrations of iron[30]. Moreover, a high level of intracellular iron in cancer cells is tolerable because of iron-dependent proteins.

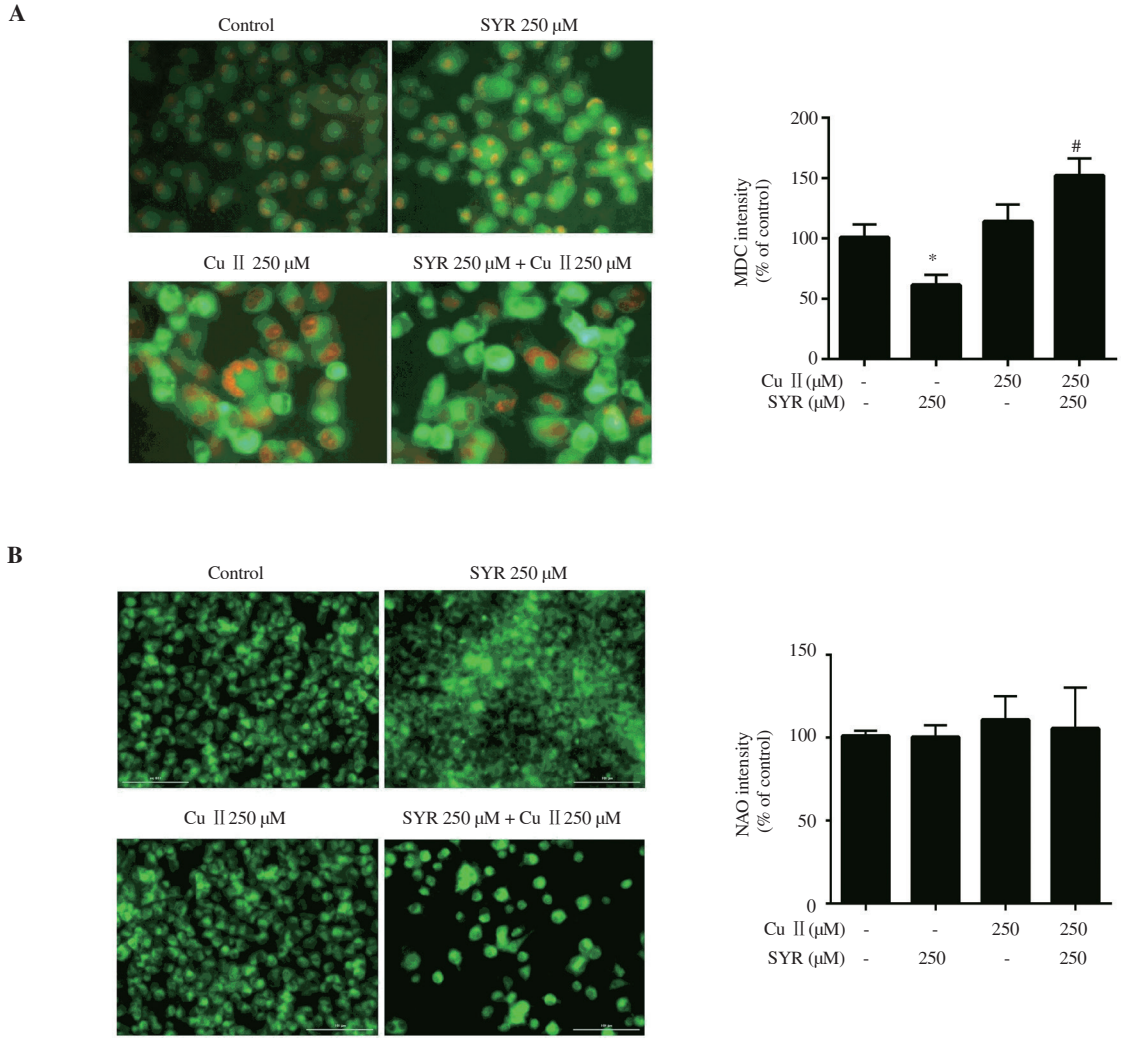

Figure 5. Effect of co-treatment with SYR and Cu (Ⅱ) on autophagy (A) and mitochondrial mass (B) in HepG2 cells. Autophagic vacuoles were determined by monodansylcadaverine (MDC) and mitochondrial mass was determined by using the fluorescent probes, NAO. $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the Cu (Ⅱ) (250 μM) .

These proteins are involved in several cellular processes such as DNA synthesis, DNA repair, cell cycle regulation, angiogenesis, and metastasis[31].

According to our results, lower concentrations of Cu (Ⅱ) in the HEK cells were toxic in comparison to the HepG2 cells after 24 h of incubation. Thus, in this study, we chose the concentrations of Cu (Ⅱ) that were non-toxic to normal cells but toxic to cancer cells at 72 h.

In MTT assay, high doses of SYR (750-1 000 μM) were toxic to the HEK cells, while these doses were not toxic to HepG2 after 24 h of treatment. However, after 72 h of incubation, 50-150 μM of SYR increased the cell viability in the HEK cell in comparison to the controls, whereas 500-1 000 μM of SYR reduced the cell viability significantly. However, none of all SYR concentrations was toxic to HepG2 cells at 72 h. This can be related to high amounts of glutathione content and cellular antioxidant in hepatic cells in comparison to renal cells. Therefore, SYR did not show any toxicity in cancer cells at high concentrations.

In agreement with our results, a low concentration of caffeic acid increased the viability of the HL-60 cells. Conversely, high concentrations of caffeic acid inhibited cell proliferation[32].

According to previous studies, phenolic compounds are pro-oxidant with cytotoxic effects in certain conditions[33-35]. Their antioxidant/pro-oxidant activity can be affected by different factors such as metal-reducing agents, high pH, and high concentration of antioxidants[36].

Treatment of the HepG2 cells with Fe (Ⅱ) + SYR for 24 and 72 h did not show any toxicity effects. It can be related to a high amount of glutathione and ferritin in HepG2 which was mentioned before. When HEK cells were incubated with Cu (Ⅱ)+SYR, cell viability was reduced, but this toxicity was not seen after 72 h incubation. It seems that extended incubation time may induce some cellular pathways that can help cells to tolerate toxic compounds but this pathway would not accrue in a short time. Treatment of HepG2 cells with Cu (Ⅱ)+SYR for 24 and 72 h also show no toxicity effects at 24 h but it was toxic at 72 h, indicating that adding SYR to Cu (Ⅱ) increased toxicity of copper and confirming pro-oxidant activity of SYR.

In colony-forming assay, treatment of HepG2 cell with Cu (Ⅱ) or SYR+Cu (Ⅱ) reduced plating efficiency and surviving fraction in comparison to the control. In agreement with previous results, $CuSO_4$ reduced the proportion of cells in G_1 with induction of apoptosis. In contrast, the cells exposed to Cu (Ⅱ) + SYR increased the proportion of cells in the sub-G_1 and G_2/M phase in comparison to the cells treated with $CuSO_4$ only. According to this result, cancer cell growth and division were arrested in the G_2/M phase.

Consistent with our results, a previous study showed that in the reaction system containing copper, catechol, and DNA, the formation of free radicals is responsible for DNA damages. These radicals were hydroxyl radicals and singlet oxygen[37]. In another study, curcumin played the role of pro-oxidant in the presence of copper; so, the oxygen molecule was activated and produced free radicals which broke the DNA strand and induced apoptotic cell death. This apoptosis was strongly related to formation of high concentrations of ROS in cells[38]. According to our results, the cancer cells were more resistant to $FeSO_4$ + SYR in comparison to normal cells. A previous study suggested that phenolic compounds were more effective in inhibiting iron-mediated DNA damages in comparison to Cu-mediated DNA damages[39].

The other reason for the pro-oxidant activity of Cu (Ⅱ)+SYR in HepG2 which did not occur in the HEK cells can be rooted in the difference in antioxidant capacity of these cells. It seems that lower levels of catalase in cancer cells lead to a lower capacity to remove H_2O_2 as H_2O_2 can be the product of Cu (Ⅱ) mediated Fenton reaction. Accordingly, cancer cells are more sensitive to metals in comparison to normal cells[35,40].

Autophagy is a preservative mechanism to remove the dysfunctional organelles and cellular components in order to regenerate cells. Various studies considered the role of abnormal autophagy in different human diseases, mainly neurodegenerative diseases and cancers[41].

Some evidence shows that pharmaceutical chemotherapeutic agents induce autophagy in cancer cells[42]. Moreover, other antitumor treatments such as hormones and ionizing radiation, may motivate autophagy and lead to apoptosis of tumor cells[43]. Previous findings showed that changes in the morphologic characteristics could be the cause of autophagic cell death. Also, the other mechanism can be related to selective degradation of catalase, ROS generation, and caspase inhibition[19].

In our study, the level of ROS in cells treated with Cu (Ⅱ) + SYR was increased significantly; moreover, the cells treated with Cu (Ⅱ)+ SYR increased vacuole-like structures in the cytoplasm when compared to control. The major morphological change of autophagic cells is the formation of autophagic vacuoles[30]. Our results indicated that in the presence of Cu (Ⅱ) + SYR, cancer cell death occurred by autophagy. It was suggested that suppressed cell proliferation *via* excessively induced autophagy could be considered in the apoptosis-resistant cancer cell treatment. However, no changes observed in mitochondrial mass and mitochondrial membrane potential may indicate that mitophagy was not responsible for cell death. In agreement with our results, galangin (flavonols, obtained from *Alpinia officinarum*), induced autophagy in HepG2 cells by p53-dependent pathway and increased the number of cells that contained vacuoles[44]. Moreover, Azmi *et al.* reported that quercetin amplified the generation of autophagosomes and autolysosomes in both *in vitro* and *in vivo* models. In a previous study, resveratrol in the presence of Cu (Ⅱ) caused DNA degradation in cells and this damage was inhibited in the presence of a ROS scavenger. So it was suggested that DNA damages increase because of ROS formation *via* reducing the Cu (Ⅱ) to Cu (Ⅰ) in the presence of polyphenols[45].

In addition, autophagy regulation is an appropriate target in developing novel therapeutic agents. The ability of polyphenol to suppress cell proliferation and apoptosis or autophagy induction can be further investigated in the future study.

To achieve these goals, it is necessary to identify the proteins that are involved in this process and realize their roles in autophagy. This part is the limitation of our study and should be noticed in our future research.

As the pro-oxidant activity of polyphenols shows their potential role in the prevention of some diseases like cancer, these bioactivities have been more considered in recent decades. Pro-oxidant activity is like two sides of a coin; in some cases, pro-oxidant activity leads to cellular damages. However, the pro-oxidant activity of natural antioxidants can be responsible for cellular regulation in malignant cells.

According to the limitation of our study, further investigations are warranted to analyze these biological activities of SYR in other types of normal and cancer cells. Also, in the next step, it is necessary to apply more methods to clarify the involved proteins in signaling pathways of autophagy and mitophagy which are affected by SYR.

In conclusion, the combination of SYR with Cu (Ⅱ) was toxic to cancer cells and showed pro-oxidant activity. Also, treatment of cells with SYR+Cu (Ⅱ) led to ROS production, apoptosis induction, and autophagy in cancer cells with less cytotoxic effects on normal cells. Thus, pro-oxidant compounds can be a proper candidate in cancer treatment. However, it is necessary to reveal their molecular mechanism in the future investigation to overcome the limitation of natural products in clinics.

Conflict of interest statement

The authors declared no conflict of interest.

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Authors' contributions

ZS and MR conceived and designed the work, analyzed data, wrote and revised the article, and finally approved the version to be published. RA conceived and designed the work, and analyzed data. AN, MSH, SHM, FI, and JS collected and analyzed data.

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