




# Isorhamnetin decreased the expression of HMG-CoA reductase and increased LDL receptors in HepG2 cells

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## ABSTRACT

Isorhamnetin is a flavonoid present in many plants. In a previous *in vivo* study, isorhamnetin lowered serum cholesterol of rats fed high-cholesterol diet. However, its mechanism of action was not investigated. In the present work, the mechanism of its hypocholesterolemic effect was studied. The expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) and low-density lipoprotein receptor (LDLR) genes and proteins was studied in HepG2 liver cancer cell line by polymerase chain reaction, western blot, and indirect enzyme-linked immunosorbent assay as well as its antioxidant activity. Isorhamnetin had an  $IC_{50}$  of 100, 53, and 40  $\mu$ M at 24, 48, and 72 hours, respectively, in HepG2 cells. Isorhamnetin downregulated HMG-CoA reductase gene expression significantly. Also, all tested doses of isorhamnetin downregulated LDLR expression and produced no change in membranous LDLR protein expression. In cell lysate, LDLR was increased by all studied concentrations of isorhamnetin. Isorhamnetin (100  $\mu$ M) decreased intracellular HMG-CoA reductase compared to vehicle-treated control. Furthermore, isorhamnetin increased superoxide dismutase activity and reduced  $H_2O_2$  level, due to catalase activity. Isorhamnetin reduced HMG-CoA reductase gene expression and increased total LDLR and exerted pronounced antioxidant action.

## INTRODUCTION

Cholesterol is an insoluble lipid molecule that provides stability to membrane bilayers and is essential for their function (Cohen, 2008). It is also a precursor for steroid hormones such as sex hormones and corticosteroids (Wang *et al.*, 2017). Despite the gastrointestinal tract's ability to absorb substantial amounts of cholesterol, almost all cells can generate their own requirement. Only the liver's hepatocytes, however, are capable of removing huge amounts of cholesterol (Cohen, 2008).

Cholesterol is transferred in the bloodstream by lipoprotein particles. Low-density lipoprotein (LDL), high-density lipoprotein (HDL), and the two main cholesterol-carrying blood lipoproteins were identified by physiologists in the 1950s and 1960s. Epidemiologists revealed that high LDL levels

increase the risk of heart attacks, while high HDL levels protect against heart disease (Goldstein and Brown, 2009). Cholesterol usually forms solid lumps in the walls of arteries when LDL levels are abnormally high, a state known as atherosclerosis, which is primarily responsible for coronary heart disease and other kinds of cardiovascular disease (Hrydziuszko *et al.*, 2014).

The main cause of morbidity and mortality worldwide is atherosclerotic cardiovascular disease (ASCVD) and its clinical manifestations, such as myocardial infarction and ischemic stroke (FERENCE *et al.*, 2017). The preservation and accumulation of cholesterol-rich apoB-containing lipoproteins within the arterial intima at plaque-prone sites are key pathophysiological mechanisms in the establishment of ASCVD (FERENCE *et al.*, 2017). Over 200 studies including more than 2 million individuals and over 150,000 cardiovascular events demonstrated a dose-dependent, log-linear relationship between LDL level and ASCVD threat (FERENCE *et al.*, 2017; Lewington *et al.*, 2007; Nordestgaard *et al.*, 2013).

Statins are commercially available drugs used to lower cholesterol level. They are well known for inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the enzyme

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that catalyzes the rate-limiting step in cholesterol biosynthesis. The therapeutic efficacy of statins for individuals with coronary artery disease and cardiovascular disease has been proven (Jiang *et al.*, 2018). They are not, however, without adverse effects, which are usually dose-related and include elevated liver enzymes and muscle pain (Grundy, 2005). Also, despite their ability to lower plasma LDL, statins were not always effective at preventing cardiovascular disease (Tavazzi *et al.*, 2008). Therefore, new drugs with less side effects and better therapeutic outcomes are needed.

Isorhamnetin is a naturally occurring methylated derivative of quercetin. Isorhamnetin is present in *Hippophae rhamnoides* L., *Ginkgo biloba* L., and *Brassica juncea* (Yokozawa *et al.*, 2002). It had a wide spectrum of pharmacological effects, including hypoglycemic (Yokozawa *et al.*, 2002), cardioprotective (Zhao and Liu, 2008), neuroprotective (Zhao *et al.*, 2016), anti-inflammation (Chi *et al.*, 2016), antitumor (Wu *et al.*, 2019), and antioxidant effects (Dong *et al.*, 2015). In an *in vivo* study, isorhamnetin lowered serum cholesterol of rats fed high-cholesterol diet (Igarashi and Ohmuma, 1995). However, its mechanism of action was not studied. This work was designed to investigate the effect of isorhamnetin on HMG-CoA reductase and LDLR gene and protein expression in HepG2 hepatoma cell line. Furthermore, the antioxidant activity of isorhamnetin in HepG2 was investigated.

## MATERIAL AND METHODS

### Drugs

Isorhamnetin stock solution was prepared by dissolving it in 10% DMSO. Stock solution was diluted by Dulbecco's Modified Eagles Medium (DMEM) media to obtain the final concentrations 25, 50, or 100  $\mu$ M used in different studies.

### Cells and culture media

Human HepG2 hepatocarcinoma (ECACC/UK) cells were cultured as monolayer using complete DMEM (Euroclone, Italy). DMEM contains four times the amino acid and vitamin concentration of original Eagle's Minimal Essential Medium. Additional supplements include 4 mM L-glutamine, 4,500 mg/l glucose, 1 mM sodium pyruvate, and 1,500 mg/l sodium bicarbonate. To DMEM medium, 10% fetal bovine serum and penicillin/streptomycin (100 U/ml) were added and incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37°C.

### Cytotoxicity assay

The toxicity of isorhamnetin was investigated using 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Promega, USA). The test was performed according to the manufacturer's directions as described earlier (Abbas *et al.*, 2020).

The MTT test (Promega, USA) is based on the ability of mitochondrial dehydrogenase to convert MTT to a purple formazan product. The antiproliferative activity of isorhamnetin (Biosynth Carboxynth, UK) (200, 100, 50, 25, 12.5, 6.25, and 3.125 g/ml) was evaluated using this method. The cells were suspended, collected, and counted to 10<sup>4</sup>/100  $\mu$ l before being seeded in a 96-well plate and incubated for 24 hours at 37°C, 5% CO<sub>2</sub>. The test was carried out in accordance with the manufacturer's instructions: in each well, 15  $\mu$ l of MTT dye solution was added and incubated for

4 hours. The MTT-formazan product was then stopped by adding 100  $\mu$ l of MTT stop solution to each well. The absorbance was then measured at 590 nm with a microplate reader (Biotech, USA); a decrease in absorbance compared to untreated control cells is a measure of cell viability. Each test was performed in triplicate and HepG2 cells without treatment were as negative control. The final concentration of DMSO in all solutions was <0.05%.

Cell viability percentage was calculated using the formula:

$$\% \text{ Cell viability} = (\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}) / (\text{OD}_{\text{Vehicle}} - \text{OD}_{\text{Blank}}) \times 100\%.$$

OD is the optical density.

### Quantitative real-time polymerase chain reaction (PCR)

HepG2 cell line (10<sup>6</sup> cells) was cultured in DMEM medium and seeded in 24 cell plates. Then, HepG2 cells were treated with less than 1% DMSO (vehicle) or isorhamnetin (25, 50, or 100  $\mu$ M) for 24 hours. At the end of incubation, cells were washed with PBS and then trypsinized. Total RNA was extracted using Direct-zol RNA MiniPrep Kit (Zymo, USA Cat# R2050) according to the manufacturer's instructions. RNA (1  $\mu$ g) was extracted from HepG2 cells treated with vehicle or isorhamnetin and used to prepare cDNA and to run real-time PCR using GoTaq® 1-Step RT-qPCR (Promega, Cat# A6020, USA). RT-PCR was performed using iCycler Thermal Cycler System apparatus (Thermo Fisher, USA) under the following conditions: 90°C for 10 seconds followed by 60°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. At the end of RT-PCR melting curve step at 55°C–95°C was done. Primers of the genes: *HMGCR* that encodes HMG-CoA reductase enzyme, *LDLR* that encodes LDLR, and *ACTB* (housekeeping gene) that encodes  $\beta$ -actin were purchased from Alpha DNA (Canada). Sequence of sense and antisense primers are shown in Table 1. Expression data were normalized using  $\beta$ -actin *ACTB* used as internal control for each sample.

The results of quantitative PCR were calculated using 2-Delta Delta C(T) method to analyze the relative changes in gene expression. This experiment was repeated twice in triplicate manner to insure that the results are reproducible.

### Protein extraction

After treatment with vehicle or isorhamnetin for 24 hours, HepG2 cells (10<sup>6</sup> cells) of each group were washed twice with PBS and incubated with lysis buffer (iNtRON biotechnology Cat. #17081, Korea) for 20 minutes at 4°C and finally centrifuged at 15,000 rpm for 5 minutes. Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific, USA). The supernatant was stored at –20°C for analysis of superoxide

Table 1. Sequence of primers.

Gene	Primer
<i>HMGCR</i>	(R) AGCCATAAGAGGAAGGCACCG
	(F) TCGGCCTTCACTCTCGATG
LDLR	(R) TCATCCATCGCTCCAGGTA
	(F) CTTACGGGCCCTGGACAAAT
<i>ACTB</i>	(R) TGAAGGTAGTTTCGTGGATGC
	(F) CGGGACCTGACTGACTACC

dismutase (SOD), catalase activity, western blot, and cellular enzyme linked immunosorbent assay (ELISA).

#### Western blot

Protein samples (30 µg) were separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel. At the end of the run, separated proteins were transferred to a nitrocellulose membrane (ThermoFisher, USA). 5% bovine serum albumin (BSA) (Bio-Techne, UK) in Tris-buffered saline (ChemCruz, USA) was used for blocking the membranes at 4°C overnight. In the next day, the membranes were incubated with 1:1000 rabbit monoclonal antibodies specific for HMG-CoA reductase (Abcam, US) for 1 hour at room temperature followed by washing 3 times for 10 minutes each using Tris-buffered saline with Tween-20. The membranes were then incubated for 1 hour with anti-rabbit secondary antibody conjugated with horseradish peroxidase (Bio-Techne, Minneapolis, MN).

#### Indirect ELISA for the detection of LDLR

HepG2 cells were cultured with vehicle or with different concentrations (25, 50, 100 µM) of isorhamnetin. ELISA plate was coated with 100 µl/well of cell lysate (10 µg/ml protein extract) in triplicates overnight at 4°C. At the end of the incubation period, the plate was blocked with 200 µl of 5% BSA (Bio-Techne, UK) in PBS for 2 hours at room temperature. After washing, anti-LDLR primary antibody (Bio-Techne, UK) (1:1,000 dilution) was added at room temperature for 1 hour. At the end of the incubation period, washing three times with 0.05% PBS-Tween was performed. One hundred microliters of 1:1,000 HRP-conjugated goat anti-mouse immunoglobulin G (Promega, USA) were added and incubated at room temperature for 1 hour. At the end of the incubation period, washing three times with 0.05% PBS-Tween was done. Then, 100 µl of TMB substrate solution (Biotech, USA) was added to each well and incubated for 15 minutes at room temperature before adding the stopping solution. The plate was finally read with ELISA plate reader (Biotech, USA) at 450 nm.

Cellular ELISA was also performed using 10<sup>4</sup> cells monolayer cultured for 24 hours in 96-well plate and treated with vehicle or with different concentrations (25, 50, 100 µM) of isorhamnetin in triplicates. Then, anti-LDLR primary antibody (Bio-Techne, UK) (1:1,000) dilution was added at room temperature, for 1 hour to bind with the target receptor on HepG2 cells. At the end of the incubation period, washing three times with 0.05% PBS-Tween was performed. The cells were then fixed with 4% paraformaldehyde and blocked with 200 µl of 5% BSA. HRP-conjugated goat anti-mouse immunoglobulin G (Promega, USA) (1:1,000 dilution) was added and incubated at room temperature for 1 hour. Then, 100 µl of TMB substrate was added to each well and incubated for 15 minutes at room temperature before the stop solution was added. Finally, the plate was read with ELISA plate reader (Biotech, USA) at 450 nm.

#### SOD activity assay

SOD activity was measured using Abcam SOD assay kit (Abcam, US, Cat.# ab65354). The test was performed according to the manufacturer's directions. Percentage change in SOD activity was calculated using the formula:

$$\% \text{ SOD activity} = (\text{SOD}_{\text{Sample}} - \text{SOD}_{\text{Control}}) / \text{SOD}_{\text{Control}} \times 100\%.$$

#### Catalase activity assay

Catalase assay test (Abcam, USA, Cat.# ab83464) was performed according to the manufacturer's directions.

#### Statistical analysis

In all tests, GraphPad Prism version 8 was used to perform one-way analysis of variance followed by Tukey's post-hoc test.  $p < 0.05$  was regarded as significant.

## RESULTS AND DISCUSSION

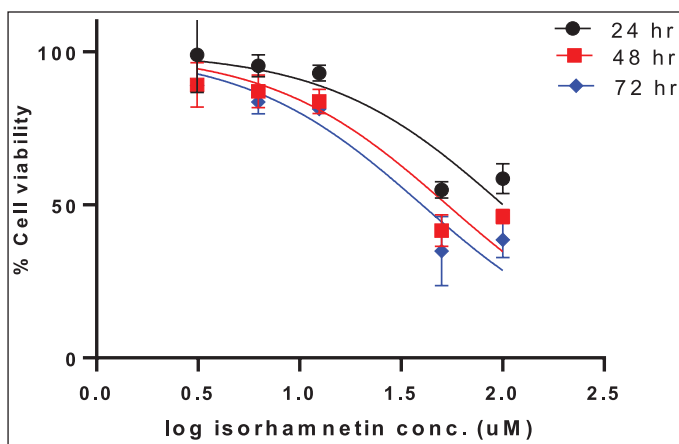
#### Cell cytotoxicity assay

In HepG2 cells, isorhamnetin exerted cytotoxic action on the HepG2 cell line with a half-maximal inhibitory concentration (IC<sub>50</sub>) value of 100 µM after 24 hours incubation, 53.3 µM after 48 hours, and 40.02 µM after 72 hours, respectively (Fig. 1). In a previous study, the IC<sub>50</sub> of isorhamnetin in HepG2 cells was reported to be 50 µM after 48 hours (Runhuan and Haiyan, 2018). This value is very close to the IC<sub>50</sub> obtained in our study. Xue *et al.* (2021) found that the IC<sub>50</sub> value of isorhamnetin was 174 µM after 72 hours of treatment in HepG2 cells. In a study performed by Gao *et al.* (2021), isorhamnetin showed proliferation inhibition on HepG2 cells with an IC<sub>50</sub> of 54.32 µM after 24 hours of treatment. Also, in another study, it was found that with the increase in isorhamnetin concentration, cell viability gradually reduced. A study of bioactivities of seven flavonoids from *Osmanthus fragrans* "Jinqiu" was done by Zhou *et al.* (2018) on seven flavonoid compounds. Isorhamnetin cell proliferation inhibition on HepG2 cells indicated IC<sub>50</sub> of 50.15 µg/ml (158 µM) after 72 hours.

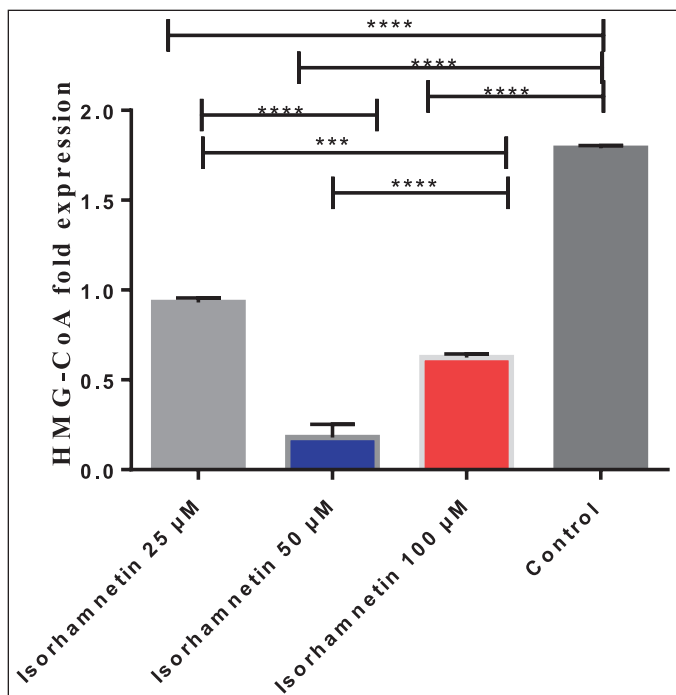
#### HMG-CoA reductase gene and protein expression

Significant downregulation of HMG-CoA reductase gene expression was produced by 25, 50, and 100 µM isorhamnetin by 48.0%, 90.0%, and 65.3%, respectively (Fig. 2). On the other hand, isorhamnetin (100 µM) decreased HMG-CoA reductase compared to vehicle-treated control (Fig. 3).

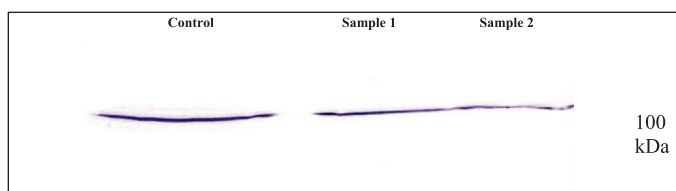
HMG-CoA reductase, the time-limiting enzyme in cholesterol biosynthesis, is a main contributor in cholesterol biosynthesis and regulation (Goldstein and Brown, 1990). Currently, HMG-CoA reductase inhibitors, such as statins, are



**Figure 1.** Cell cytotoxicity after treatment with isorhamnetin at 24, 48, and 72 hours.



**Figure 2.** Gene expression of *HMGCR* reductase enzyme in vehicle-treated and isorhamnetin-treated HepG2 cells. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ .

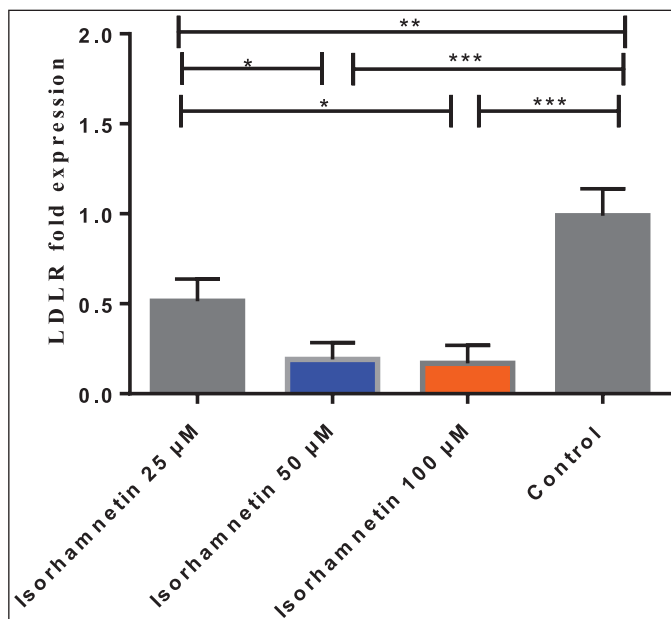


**Figure 3.** Immunoblot of HMG-CoA reductase enzyme. Control: Extract of HepG2 treated with DMSO. Sample 1: extract of HepG2 treated with isorhamnetin 25 µM. Sample 2: extract of HepG2 treated with isorhamnetin 100 µM.

commonly used to lower blood cholesterol level (Reiner, 2010). In our study, isorhamnetin decreased HMG-CoA reductase gene expression in HepG2 cells. Our results agree with earlier reports that isorhamnetin extracted from sea buckthorn decreased HMG-CoA reductase gene expression in a dose-dependent manner in HL7702 human normal liver cell line (Xiao *et al.*, 2021). Also, a decrease in HMG-CoA reductase gene expression was reported in high-fat diet-induced nonalcoholic fatty liver disease model in rats (Zhang *et al.*, 2013). Furthermore, it was demonstrated that isorhamnetin was able to activate AMP-activated protein kinase (AMPK) in HepG2 cells (Dong *et al.*, 2014). This enzyme phosphorylates and inhibits SREBP-2, a transcription factor that binds to the promoter region of HMG-CoA reductase to regulate its gene expression (Li *et al.*, 2011).

#### LDLR gene expression and protein expression in tissue homogenate and on cell surface

Cellular ELISA was used in this work to measure the surface expression of the receptor while ELISA for the whole cell extract was used to measure the total concentration of the receptor



**Figure 4.** Gene expression of *LDLR* in vehicle-treated, and isorhamnetin-treated HepG2 cells. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ .

either that was expressed on the surface or present in the cytoplasm (Pandey *et al.*, 2019). Isorhamnetin downregulated LDLR expression by 47.9%, 80.6%, and 82.7% using 25, 50, and 100 µM doses, respectively (Fig. 4). In tissue homogenate, isorhamnetin produced an increase in total LDLR protein expression at all studied concentrations (Fig. 5a). On the other hand, isorhamnetin produced no change in membranous LDLR protein expression on cell surface at all studied doses (Fig. 5b).

LDL cholesterol clearance from cells to the liver is crucial to avoid the formation of atherosclerotic lesions. In fact, about 75% of the circulating cholesterol is removed by LDLR endocytic circulation (Yang *et al.*, 2020). It is well established that statins—the most commonly used antihypercholesterolemic drugs—upregulate LDLR (Vogel, 2012). In the present study, LDLR gene expression decreased after treatment of HepG2 cells with isorhamnetin. Similar results were obtained in a previous study on normal human liver cells (HL7702) where isorhamnetin—extracted from sea buckthorn—downregulated LDLR and SREBP-2 gene expression (Gu *et al.*, 2022). Membranous LDLR was not affected by isorhamnetin treatment after 24 hours in the present investigation. On the other hand, total LDLR, including the intracellular LDLR, was higher compared to the control. One possible explanation is that isorhamnetin might decrease the degradation of LDLR. Detailed investigation of the effect of isorhamnetin on inducible degrader of the LDLR (IDOL) and proprotein convertase subtilisin/kexin type 9 pathways, involved in LDLR degradation, in HepG2 is needed. It is worth mentioning that isorhamnetin downregulated liver X receptor (LXR- $\alpha$ ) gene expression in 3T3 cell line (Lee *et al.*, 2009). LXR is a transcription factor that induces the expression of IDOL, a protein that triggers ubiquitination of the receptor and targets it for degradation (Zelcer *et al.*, 2009). If LXR was downregulated, it is expected that LDLR will increase.

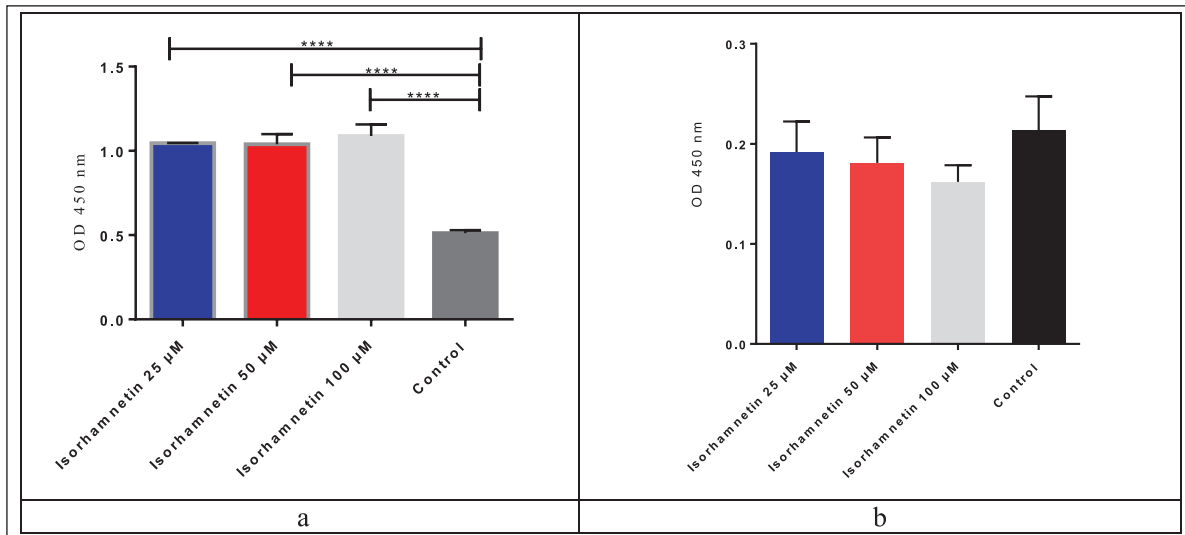
Another suggested mechanism for the effect of isorhamnetin on LDLR accumulation inside the cells is through

the activation of AMPK, the enzyme that phosphorylates and inhibits SREBP-2 (Dong *et al.*, 2014). It is well established that LDLR gene is regulated by the nuclear form of nSREBP-2 (Attie and Seidah, 2005). Therefore, investigating the signaling pathway AMPK/SREBP-2/LDLR is required.

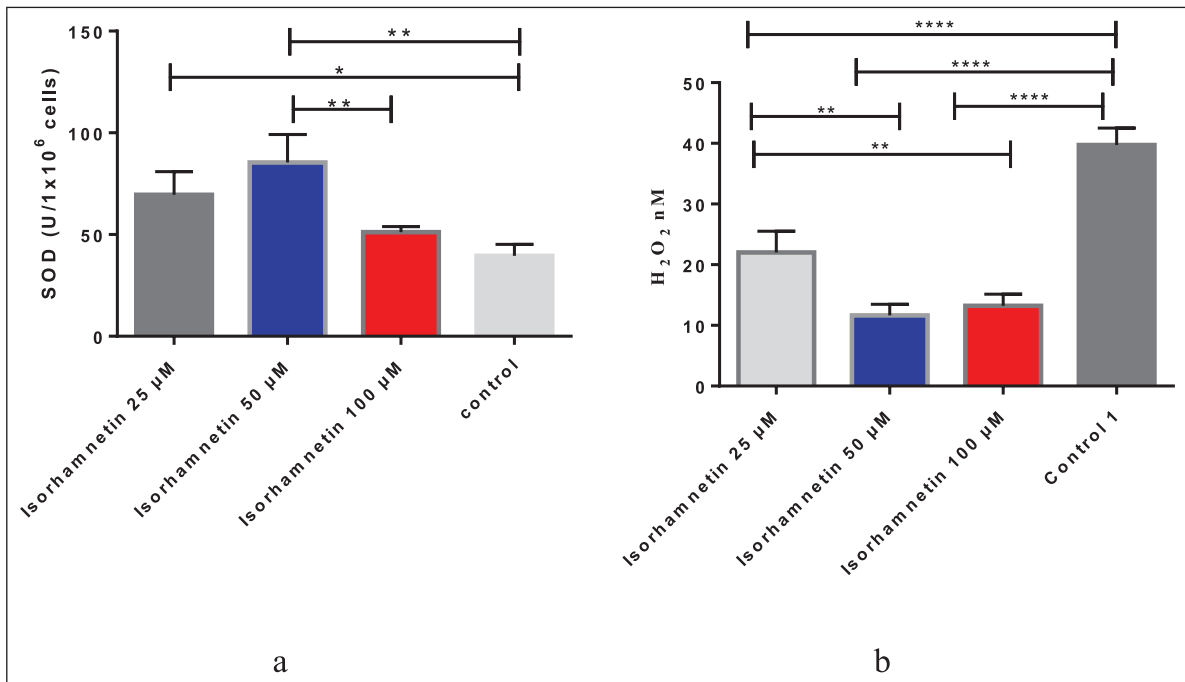
### Antioxidant activity

Isorhamnetin 25 and 50  $\mu\text{M}$ , but not 100  $\mu\text{M}$ , increased SOD activity compared to the vehicle-treated cells (control) significantly by 75.66% and 115.69%, respectively (Fig. 6a).

Similarly, isorhamnetin 25, 50, and 100  $\mu\text{M}$  reduced  $\text{H}_2\text{O}_2$  level significantly by 46.44%, 72.30%, and 66.67%, respectively (Fig. 6b). This study demonstrated that isorhamnetin decreased oxidative stress in HepG2 cell line. SOD activity increased by 2.16 folds by 50  $\mu\text{M}$  isorhamnetin treatment. Similarly, catalase activity showed a decrease in  $\text{H}_2\text{O}_2$  level (by 3.42 folds using 50  $\mu\text{M}$  isorhamnetin). In HepG2, isorhamnetin containing extract of *Tamarindus indica* leaf increased the activity of SOD and catalase (Razali *et al.*, 2015). Also, isorhamnetin enhanced antioxidant effect in different tissues including heart, brain,



**Figure 5.** Detection of LDLR in HepG2 cell homogenate (a) or expressed in the cell membrane by cellular ELISA (b) after treatment with vehicle or different doses of isorhamnetin. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ .



**Figure 6.** (a) SOD activity after isorhamnetin treatment. (b)  $\text{H}_2\text{O}_2$  reduction after isorhamnetin treatment which reflects catalase activity. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ .

and testis. Isorhamnetin exerted cardioprotective effect against myocardial ischemia-reperfusion injury in isolated rat heart and increased SOD and catalase activity (Xu *et al.*, 2020). In a study of the effect of isorhamnetin in brain, isorhamnetin improved cognition and memory, presumably via improving the antioxidant defense system (SOD and catalase activity), cholinergic signaling, and synaptic plasticity. Pretreatment of mice with isorhamnetin before scopolamine produced dose dependency and significant increase in SOD activity in the hippocampus (2.95 folds) and prefrontal cortex (2.26 folds) compared with vehicle-scopolamine-treated group. Similarly, pretreatment of mice with isorhamnetin produced dose dependency and a significant increase in catalase activity in the hippocampus (2.41 folds) and prefrontal cortex (2.63 folds) when compared with vehicle-scopolamine-treated group (Ishola *et al.*, 2019). Mustafa *et al.* (2022) studied the effect of isorhamnetin in doxorubicin-induced testicular injury model in male rats. Doxorubicin-induced oxidative stress was significantly reduced by isorhamnetin treatment due to the increase in SOD (by 2.39 folds) and catalase (by 1.72 folds) activities compared to doxorubicin-treated control (Mustafa *et al.*, 2022).

## CONCLUSION

Isorhamnetin lowered the expression of HMG-CoA reductase. Also, it increased LDLR expression in HepG2 cells. Furthermore, this flavonoid exerted antioxidant effects by increasing the activity of SOD and catalase. Future preclinical and clinical studies are needed to investigate thoroughly the effect of this flavonoid on cholesterol and lipid metabolism and to study its toxicity *in vivo*.

## Limitations of the study

To follow up gene and protein expression precisely, frequent testing is required at different time intervals. The time for performing each experiment was based on pilot studies. Frequent sampling was not possible.

## AUTHORS' CONTRIBUTIONS

This is a part of Randa Al-Rayyes's Msc. thesis; she contributed to the practical work. Manal M. Abbas contributed with study design, writing the manuscript, and statistical analysis. Razan Obeidat performed PCR, ELISA, and Immunoblot experiments.

## FINANCIAL SUPPORT

There is no funding to report.

## CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest.

## ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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