Biochemistry and Biophysics Reports 35 (2023) 101521

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Ferulic acid protects HepG2 cells and mouse liver from iron-induced damage

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ARTICLE INFO

Keywords:
Ferulic acid
Iron
Oxidative stress
Nuclear factor erythroid-2-related factor 2

ABSTRACT

Liver as iron storage organ is particularly susceptible to oxidative stress-induced injury from excess iron. Thus, antioxidant therapies are often used to reverse oxidative damage and protect cells and tissues. This study investigated the protective effects of phenolic acids; ferulic acid (FA) and its metabolite, ferulic acid 4-O-sulfate disodium salt (FAS) against oxidative stress under iron overload conditions in mouse and HepG2 cells. Cells were exposed to FA or FAS and then treated with iron-induced oxidative stress complex of 50 μmol/L FAC and 20 μmol/L of 8-hydroxyquinoline 8HQ (8HQ-FAC). Iron dextran was injected intraperitoneally on alternate days for 10 days to induce the iron overload condition in BALB/c mice. The study revealed that the phenolic acids were protective against ROS production, lipid peroxidation and antioxidant depletion in HepG2 cells and liver tissues of BALB/c mice during iron-induced oxidative stress. The protective function of phenolic acids was achieved by the transcriptional activation of nuclear factor erythroid-2-related factor 2 (Nrf2) to regulate antioxidant genes. In conclusion, the study provides evidence that FA has the potential as a therapeutic agent against iron-related diseases such as T2D.

1. Introduction

Iron is vital for several physiological enzymes, proteins, and metabolic functions. However, excess deposition of iron in the body is toxic and this could cause cell and tissue injuries [1]. Reactive oxygen species (ROS) products, which include the highly reactive hydroxyl radical (OH•) generated via the Fenton reaction damages DNA, proteins, and membrane lipids. An imbalance between ROS levels and exposure to the antioxidant detoxification system leads to damage to healthy cells and tissues. Thus, causing organ disorders such as diabetes, cancer, and neurodegenerative disease [2].

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH-$ (Fenton reaction)

In cases of elevated iron levels, iron is stored in several organs such as the spleen, liver, and kidney [2,3]. Moreover, the liver is a locus for the transformation and detoxification of xenobiotics from the body and a dysregulation of this function could cause inflammation, tissue damage and fibrosis [4]. Non-alcoholic fatty liver disease (NAFLD) is characterized by tissue iron accumulation and hepatic insulin resistance in about 30% of patients [5]. Consequently, iron depletion by chelators or phlebotomy have been investigated to alleviate iron-induced cell or tissue damage [6]. The commonly available iron chelators, deferasirox, deferoxamine, and deferiprone exhibit complications and adverse reactions. Consequently, novel treatment options that are not debilitating to iron overload patients are desirable. For example, ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA) is commonly found in several herbs, especially in traditional Chinese medicine. Phenolic compounds have protective roles in coronary heart diseases, cancer, neurodegenerative diseases, and inflammation as potential therapeutic agents [7]. Due to the hydroxyl and phenoxy groups in FA, it is a strong antioxidant that can neutralise nitric oxide and hydroxyl radical groups and scavenge electrons. Thus, FA functions in the prevention of DNA damage, cell death and oxidative stress. Moreover, it is known that FA can attenuate oxidative stress via the Nrf2 antioxidant gene [8].

The activation of the transcription factor Nrf2 (nuclear factor

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https://doi.org/10.1016/j.bbrep.2023.101521
Received 30 March 2023; Received in revised form 5 July 2023; Accepted 25 July 2023
Available online 31 July 2023

erythroid 2-related factor 2) is a cellular defence mechanism against oxidative stress. Nrf2 plays a major role in antioxidant defence mechanisms by regulating downstream detoxifying and antioxidant genes, such as NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), cystine/glutamate antiporter (SLC7A11), glutathione peroxidase 4 (GPX4) and glutamate-cysteine ligase catalytic subunit (GCLC). Therefore, the proper function of Nrf2 is essential for the survival of cells against oxidative stress-related damage by deploying antioxidant enzymes that maintain the intracellular redox status [9].

Production of ROS increases in the liver due to toxicity caused by excess iron accumulation [10]. To characterize the relationship between phenolic acids and excess iron, the study evaluated in vitro and in vivo the mitigation of phenolic acids on iron-related oxidative stress in HepG2 cells and liver tissues. Therefore, elucidating the effect of phenolic acids such as FA and ferulic acid 4-O-sulfate disodium salt (FAS) on liver oxidative metabolism is vital in identifying natural antioxidant therapies [11].

Furthermore, possible regulatory mechanisms that underlie the functions of FA, against iron-induced toxicity in both HepG2 cells and in liver tissues by the Nrf2 activation were investigated. In addition, the antioxidant response processes such as the MDA, GSH, SOD levels in cells and liver tissue were determined. The study revealed the vital function of Nrf2 in the protection of cells against iron toxicity through the upregulation of multiple antioxidant genes (NQO1, GCLC, HO-1, GPX4) that are involved in ROS metabolism. Collectively, these results indicate that FA serves as a potent antioxidant against iron-induced injury via the Nrf2 activation.

2. Materials and methods

The following reagents were purchased from the indicated commercial sources. The antibody to GPX4 was obtained from R&D systems (Abingdon, UK) while Nrf2 was purchased from Santa Cruz Biotechnology (Wembley, UK). The antibodies to FPN, NQO1, GCLC, HO-1 and β-Actin were obtained from Abcam (Cambridge, UK). All other reagents were procured from Sigma Aldrich (Dorset, UK) unless otherwise specified. Moreover, the methods employed in the current study are standard operating procedures as described in our previous studies [12,13].

2.1. HepG2 cells

HepG2 cells (purchased from ATCC) were maintained in Eagle's minimal essential medium (MEM) (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, UK), 1% glutamine (Gibco, UK), 1% penicillin–streptomycin (Sigma-Aldrich, UK) and 1% non-essential amino acids (Sigma-Aldrich, UK). Cells were stored at 37 °C under a humidified incubator including 5% CO_2. The medium was changed two-three times in a week. Cells split or were used for experiments when they were 80–90% confluent. For experiments, HepG2 cells were exposed to iron and/or phenolic acids over time periods indicated below. Control groups were treated with 0.01% DMSO only.

2.2. Iron-induced stress on HepG2 cells

HepG2 cells were pre-treated with phenolic natural compounds before were exposed to rapid iron overload damage [14] with 50 μmol/L FAC and 20 μmol/L 8HQ (8HQ-FAC). Cells were incubated for a further 2 h at 37 °C to allow quick iron accumulation. Exposure to 8HQ-FAC for 2 h (or less) is sufficient to increase cellular iron content, decrease cell viability and induce cell death [14,15]. Afterwards, cell viability was taken place with the MTT assay.

2.3. Cell viability assay

Ameliorative effects of phenolic acids, FA and FAS against iron-induced cell death were analysed in HepG2 hepatoma cells. HepG2 cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay in a 96-well plate. Briefly, HepG2 cells were seeded at a density of 5×10^3 cells per well and pre-treated with different concentrations of phenolic acids (FA or FAS) overnight, before exposure to 8HQ-FAC for 2 h. The control group was exposed with 0.01% DMSO only. Then, this was used to normalize the effects of the treatment groups, expressed as percentage. Afterwards, 100 μL of fresh MEM containing 10 μL of MTT solution (5 mg/mL in fresh sterile phosphate buffer saline) was added to each well. After incubation for 3 h at 37 °C, the dark blue formazan crystals formed in intact cells were extracted with 100 μL of DMSO and incubated for 20 min at room temperature. Optical density was read at 490 nm excitation in a microplate reader (Bio-Tek ELx800) to determine MTT reaction in the cells. Cell viability was shown as a percentage of the controls [16].

2.4. Determination of intracellular reactive oxygen species

According to the manufacturer's recommendations, (H2DCF) cell-permeant probe was used to detect intracellular reactive oxygen species in HepG2 cells. Briefly, HepG2 cells from different treatment groups were collected and rinsed three times with fresh sterile PBS. Afterwards, they were incubated for 90 min in the dark at 37 °C in PBS including 10 μmol/L of H2DCF and washed three times with sterile PBS to remove the extracellular DCFH-DA. The level of ROS into cells was examined by flow cytometry-based on the fluorescence intensity of DCF at 525 nm after a response to 485 nm excitation. The levels of ROS were shown as units of fluorescence compared with that of the control group (0.01% DMSO).

2.5. Animals

BALB/c male mice, 6 weeks old and 24–30 g each were housed at 21–23 °C in 12 h dark/light cycle. The animals were fed with a standard laboratory pellet diet and water ad libitum. Animal care and all procedures were conducted in accordance with methods approved by the College Research Ethics Committee of King's College London, and the United Kingdom Animals (Scientific Procedures) Act. 1986 (ASPA).

2.6. Experimental design

Six-week-old BALB/c male mice were divided randomly into seven groups of 5 animals per group, (1) Control, (2) Iron dextran (ID), (3) FA, or (4) FAS, (5) DMSO, (6) FA + ID, (7) FAS + ID. Iron treatment received five doses of ID injected intraperitoneally (i.p.) on alternate days over the 10 days to induce the iron overload condition. FA or FAS phenolic acids were given to mice daily for 10 consecutive days by gavage beginning a day before the commencement of iron loading. The untreated control mice were given sterile saline by gavage for 10 days. Euthanasia of the mice was performed on the 11th day by injection with 0.4–0.6 mL of pentobarbitone sodium (20% w/v) solution (i.p.). Liver tissues were collected and flash-frozen with liquid nitrogen and kept at −70 °C.

2.7. Determination of non-heme iron

Mice liver tissue samples were washed and weighed (1:5 wt:vol) in 0.15-mol/L NaCl in 10-mmol/L NaOH-Hepes buffer at pH 7.0 with use of a 1-mL glass Dounce homogenizer (Wheaton Scientific). Then, non-heme-iron content was analysed in an aliquot of the homogenate for with the use of the ferrozine-based colourimetric assay developed by Simpson and Peters [17]. The iron amounts were expressed as either concentration (nmol Fe/mg wet weight) or content (μmol Fe/organ).

2.8. Lipid peroxidation (MDA)

Lipid peroxidation in HepG2 cells or tissues was measured by the

detection of the endpoint product malondialdehyde (MDA) according to the manufacturer's instructions, using the MDA microplate assay kit from Cohesion Biosciences (London, UK). MDA of supernatant from cell and tissue samples were measured by colorimetry analysis at 532 and 600 nm. MDA levels were normalized based on protein content and expressed as nmol/mg protein.

2.9. Glutathione analysis (GSH)

The GSH concentration in HepG2 cells or liver tissues was measured by using a glutathione assay kit purchased from Sigma Aldrich (Dorset, UK) based on the manufacturer's instructions. The absorbance of cell and tissue homogenates was read at 412 nm at 1 min intervals 5 times. The activity level of GSH enzyme was shown as nmol/ml in samples of both cells and tissues.

2.10. CuZn-superoxide dismutase (SOD)

Superoxide dismutase activity in cells or tissues was measured using the Amplite™ Colorimetric Superoxide Dismutase (SOD) Assay Kit according to the manufacturer's instructions. The supernatants of cell and tissues samples were kept at room temperature for 45 min and the absorbance was read at 560 nm.

2.11. Western blot analysis

Liver tissues were homogenized in Radio Immunoprecipitation Assay (RIPA) lysis buffer containing 0.1 mM EDTA, 20 mM KH2PO4, 135 mM KCL, supplemented with Protease Inhibitor Cocktails (Thermo Scientific, Dartford, UK). In homogenized samples, protein concentration was measured using Bio-Rad reagents (BioRad, Munich, Germany). 20 μg protein of tissue samples were loaded on sodium dodecyl sulfate polyacrylamide gels (12% gels (BioRad, Munich, Germany)) and were transferred onto PVDF membranes. The membranes were probed with primary antibodies; GPX4 antibody (R&D systems, Abingdon, UK), FPN, GCLC, NQO1, HO-1 (Abcam, Cambridge, UK), Nrf2 (Santa Cruz Biotechnology, Wembley, UK). As the internal control, β-Actin (Thermo Scientific, Dartford, UK) was used. Then incubation with HRP-conjugated secondary antibody (diluted 1:5000, R&D Systems, Abingdon, UK) for 1 h at room temperature. Cross-reactivity signals were visualised with peroxidase-linked anti-IgG by using Clarity Western ECL Substrate (Watford, UK). The images were quantified using ImageJ software (NIH, Bethesda, MD, U.S.A.) and normalized to respective loading controls.

2.12. Real-time PCR analysis

Total RNA was isolated using the Trizol reagent (Thermofisher Scientific, UK) from liver tissues from all groups of mice. Then, RNA concentration was measured spectrophotometricaly using nanodrop, Hellma Tray-Cell Type 105.810 (Hellma Analytical). For each sample to analyse, 1 μg of RNA from each sample was converted into cDNA by Thermo Scientific Verso cDNA synthesis kit (Thermo Fisher Scientific, UK). After completing all the cycles, the DNA reaction system was run at 42°C for 50 min and then 95°C for 5 min. The described selected genes using gene specific primers were used in Table 1. It is precise that the amplified products were not from contaminating genomic DNA, a control without reverse transcriptase was also performed. Products were not observed in the minus-reverse transcriptase control sample; therefore, the sample was free from genomic DNA contamination.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, USA) with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-hoc test to compare the means of the experimental groups. Data show duplicate measurements from three independent experiments for cell-based assays and five independent experiments for animal studies. All the values are expressed as mean ± SEM. $p < 0.05$ was regarded statistically significant when concentrating on differences between groups.

Table 1
The RT-PCR specific primers.

Genes	Forward primer	Reverse primer
NFR2 (NM_010902)	catgatggacttggagttgc	cctccaaaggatgtcaatcaa
GPx4 (AB030643)	tttcctgacacagggttcact	cagcctggtctggtaagca
HO-1 (NM_010442)	agggtcaggtgtccagagaa	cttccagggccgtgtagata
NQO1 (BC004579)	agcgttcggtattacgatcc	agtacaatcagggctcttctcg
GCLC (BC019374)	agatgatagaacacgggaggag	tgatcctaaagcgattgttcttc
STAT3 (U06922)	gttcctggcaccttggatt	caacgtggcatgtgactctt
SOCS3 (BC052031)	atttcgcttcgggactagc	aacttgctgtgggtgaccat
FoxO1 (BC152908)	cttcaaggataagggcgaca	gacagattgtggcgaattga
FPN (AF215637)	gggtttcttagaagcaggtatgc	ttctcagtgtacacacctattcaagtc
FtH (NM_010239.2)	gctgaatgcaatggagtgtg	cagggtgtgcttgtcaaaga
RPL19 (NM_009078)	ccacaagctctttccttttcg	ggatccaaccagaccttcttt

3. Results

3.1. Effect of phenolic acids against iron-induced damage in HepG2 cells

Iron overload was taken place with FAC and the lipophilic iron chelator 8HQ. FAC is an extremely stable form of ferric citrate, a sorts of free iron found in blood that increases in hereditary hemochromatosis [18]. FAC-8HQ complex was used to determine iron loading into cells because 8HQ is a lipophilic chelator that facilitates rapid entry of iron into cells to exert toxicity [19]. The cytotoxicity of rapid iron overload on HepG2 cells was determined using the MTT-cell viability assay. Cells were treated with the iron complex (50 μM FAC-20 μM 8HQ) for 2 h 8HQ-mediated iron loading effects reduced cell viability by 48% compared with the untreated control group (Fig. 1). To investigate the protective effects of phenolic acids against 50 μM FAC-20 μM 8HQ induced toxicity, MTT assay was conducted to determine cell viability. HepG2 cells were exposed to 5 different levels of FA or FAS at 5, 10, 20, 30 and 40 μM overnight and then exposed to 50 μM FAC-20 μM 8HQ for 2 h. This study shows that cell viability was significantly increased at 10 μM FA or FAS after pretreatment of 50 μM FAC-20 μM 8HQ compared with the 50 μM FAC-20 μM 8HQ group ($p<0.05$) (Fig. 1A and B). Consequently, the effective concentrations of both phenolic acids at 10 μM FA or FAS and 50 μM FAC-20 μM 8HQ were selective for further experiments.

3.2. Protective functions of phenolic acids in iron treated HepG2 cells

To verify if phenolic acids can attenuate FAC-induced oxidative stress, DCFH-DA probe was used to determine ROS levels in HepG2 cells. As shown in Fig. 2A, a significant increase of ROS levels was stated in HepG2 cells after treatment with 50 μM FAC-20 μM 8HQ for 2 h ($p<0.05$). However, pre-treatment of HepG2 cells with 10 μM FA or FAS for overnight suppressed the increase of ROS compared with the 8HQ-mediated iron treatment group. These results revealed that phenolic acids could attenuate the intracellular ROS accumulation ($p<0.05$) in the cells. Furthermore, as shown in Figs. 2B and 50 μM FAC-20 μM 8HQ treatment increased MDA production compared with the control group ($p<0.05$) whereas 10 μM FA or FAS pre-treatment of similarly treated HepG2 cells significantly decreased MDA production compared with the iron treatment group. It was next determined whether FA or FAS pre-treatment can alleviate iron-induced ROS generation by the induction of the expression of antioxidant genes and proteins. GSH and SOD are important endogenous antioxidants, which play fundamental roles in protecting cells from oxidative damage [20]. As shown in Fig. 2C and D, the levels of GSH and SOD in HepG2 cells were significantly decreased

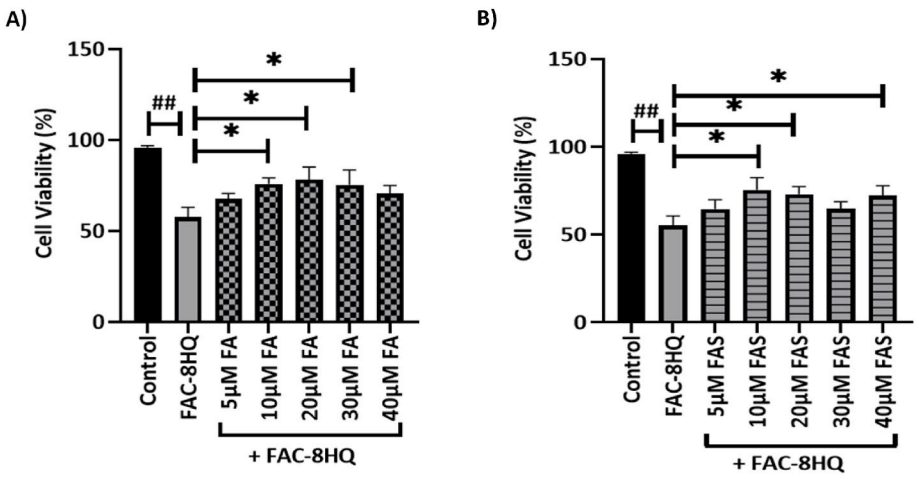

Fig. 1. Determination of dose of phenolic acids to prevent iron-induced HepG2 cell damage. HepG2 cells were supplemented with ferulic acid (FA) (A) or, ferulic acid 4-O-sulfate disodium salt (FAS) (B) (5, 10, 20, 30 and 40 μM) at the dose dependent manner for overnight. Then, cells were treated with 50 μM ferric ammonium citrate and 20 μM 8-hydroxyquinoline (FAC-8HQ) for 2 h to implement rapid iron entry into cells and cell viability was assayed. Data are presented as the mean ± SEM. n=3. $^{\#}p< 0.05$, $^{\#\#}p<0.01$ control vs. treatment groups, *p<0.05 FAC-8HQ only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

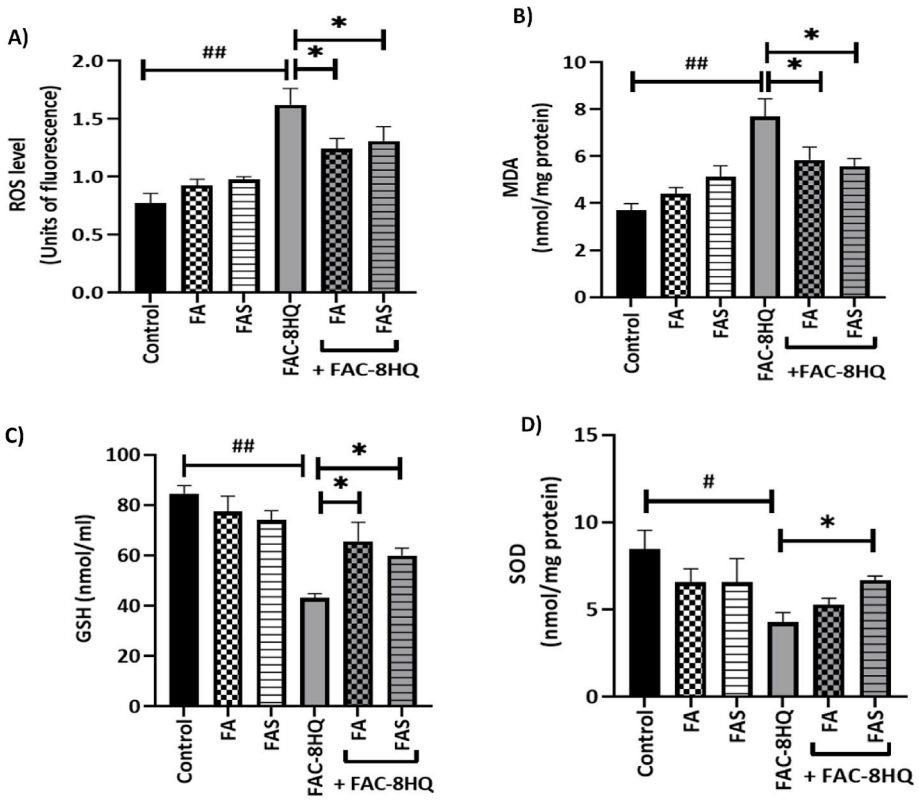

Fig. 2. Effect of phenolic acids on the activity of oxidative stress and antioxidant enzymes. HepG2 cells were exposed to 20 μM ferulic acid (FA) or, 20 μM ferulic acid 4-O-sulfate disodium salt (FAS) for overnight. Then, cells were supplemented with 50 μM ferric ammonium citrate and 20 μM 8-hydroxyquinoline (FAC-8HQ) for 2 h to implement rapid iron entry into cells. Afterwards, cellular reactive oxygen species (ROS) (A), lipid peroxidation levels (B), glutathione (GSH) amounts (C) and cellular superoxide dismutase amounts (SOD) (D) were assayed. Data are presented as the mean ± SEM. n=3. $^{\#}p<0.05$, $^{\#\#}p<0.01$ control group vs. treatment groups, *p<0.05 FAC-8HQ group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

(p<0.05) when exposed to 50 μM FAC-20 μM 8HQ in contrast to untreated control group. However, 10 μM FA and FAS pre-treatment increased GSH levels in HepG2 cells exposed to 50 μM FAC-20 μM 8HQ (p<0.05). Besides, Fig. 2D showed that SOD levels were increased by FAS pre-incubation in HepG2 cells exposed to rapid iron entry compared with iron treated group.

3.3. Attenuation of liver tissue iron accumulation by phenolic acids

Condition of iron overload was elucidated by ID injections in in vivo studies, that mimic parenteral administration of iron supplements. ID is ferric hydroxide dextran complex that is degraded in Kupffer cells of macrophages to liberate iron that is effluxed into circulation by ferroportin (FPN) [21]. Iron accumulation in the tissues was evaluated with the non-heme iron assay, to investigate if FA or FAS could influence the iron deposition levels in the liver. Experimental groups subjected to iron overload in the liver exhibited increased levels of non-heme iron by about 2-fold. However, treatments of phenolic acids, FA and FAS, appreciably reduced iron content in the liver (Fig. 3).

3.4. Antioxidant functions of ferulic acid in the liver tissue of mice

Free radicals are transient and rapidly graded such that oxidative stress status is often evaluated by measuring the endogenous antioxidant defence such as superoxide dismutase (SOD) and glutathione (GSH) [22]. Antioxidant capacities were evaluated by measuring the levels of GSH (Fig. 4A) and SOD (Fig. 4B), in the liver tissues. Levels of reduced glutathione (GSH) were increased with the treatment of FA or FAS in iron overloaded mice compared with only iron overloaded mice in the liver (Fig. 4A). Moreover, SOD levels were measured in the liver tissue. It

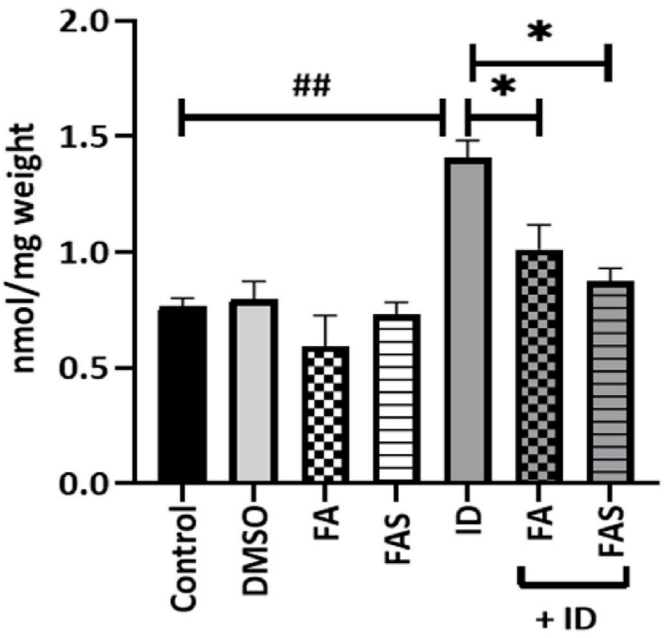

Fig. 3. Non-heme iron levels in the liver tissue of BALB/c mice. Tissue non-heme iron per gram weight tissue was measured in liver in BALB/c male mice. Data are presented as the mean ± SEM, n=5 mice per group. $^{\#}p<0.05$, $^{\#\#}p<0.01$ control group vs. treatment groups, *p<0.05 ID group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

showed that ID treatment group revealed a reduction in SOD activity in comparison to the control group in the tissues (Fig. 4B). In general, FA administration in ID groups significantly recovered SOD levels compared with ID only groups of liver tissues (Fig. 4B). As a marker of tissue damage, lipid MDA levels in the liver was determined. The MDA level in the liver was highest in ID alone treatment group ($p<0.05$) (Fig. 4C); however, FA or FAS treatments in the ID treatments displayed a statistically significant decrease in the MDA levels in the liver compared with ID alone group ($p<0.05$) (Fig. 4C).

3.5. Effects of phenolic acids on antioxidant pathway in liver tissue

To demonstrate that protective effect of phenolic acids on iron-induced oxidative stress is due to Nrf2 activation, its downstream genes and proteins were determined by qPCR and Western blot respectively in mouse liver tissue. As shown in Fig. 5 (B, C), the protein levels of total Nrf2 and its downstream antioxidative genes including NQO1, GCLC, HO-1 and GPX4 were significantly increased in iron overloaded liver after treatments with phenolic acids. In agreement with the changes of protein levels in the liver, the mRNA expressions of total Nrf2 and GPX4 in phenolic acid-treated groups in ID loaded tissues were significantly elevated ($p<0.05$) (Fig. 5A) as determined by qPCR. To explain the possible effect of FA on iron efflux, mRNA and protein levels of FPN were determined in liver tissues of mice. FPN presented a marginal increase of mRNA and protein levels in the iron overloaded groups treated with both phenolic acids in comparison with the liver tissues of ID only ($p<0.05$) (Fig. 5A and B). Also, to assess whether phenolic acid treatments might alleviate cellular iron levels, mRNA level of the iron storage protein ferritin (FtH) was measured that is highly regulated by cellular iron amounts. mRNA levels of FtH were significantly increased in the liver of iron-treated BALB/c mice ($p<0.05$), whereas phenolic acid supplementations in ID-treated mice reversed this effect significantly ($p<0.05$).

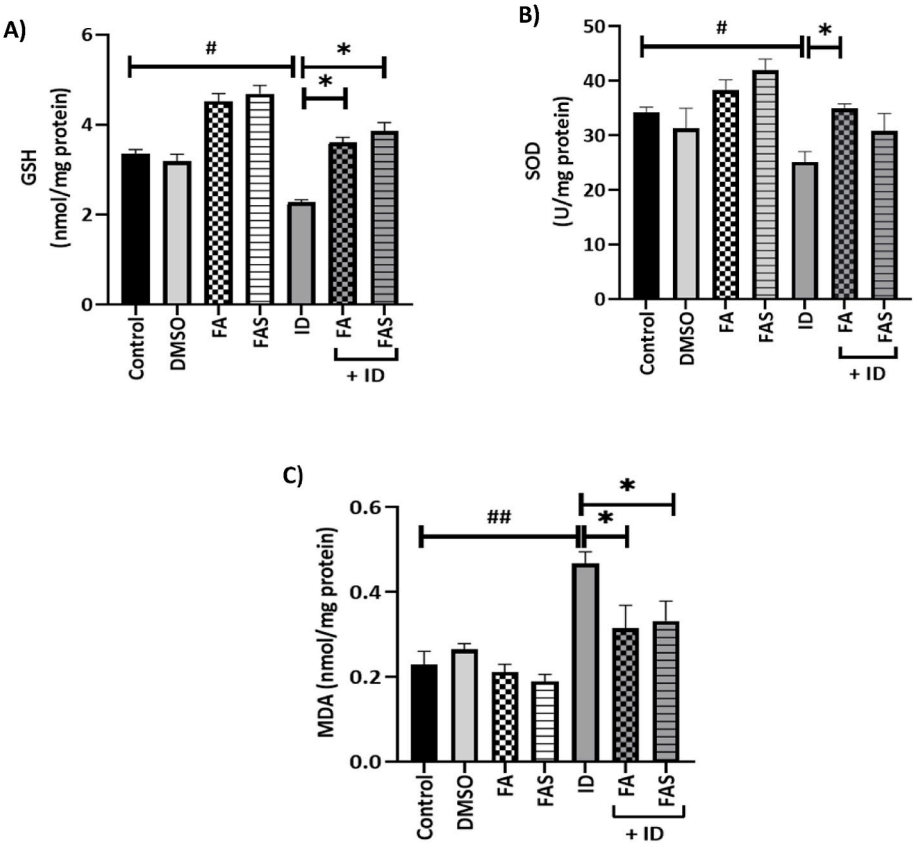

Fig. 4. Protective effects of phenolic acids against oxidative stress in the iron overloaded-liver tissues. GSH (A), Zn–Cu superoxide dismutase (SOD) activity (B), and MDA levels (C) in liver of BALB/c male mice were measured by commercial assays. Antioxidant activities in the liver tissues treated with phenolic acids were significantly different from the control group. Data are presented as the mean ± SEM, n=5 mice per group. $^{\#}p<0.05$, $^{\#\#}p<0.01$ control group vs. treatment groups, *p<0.05 ID group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

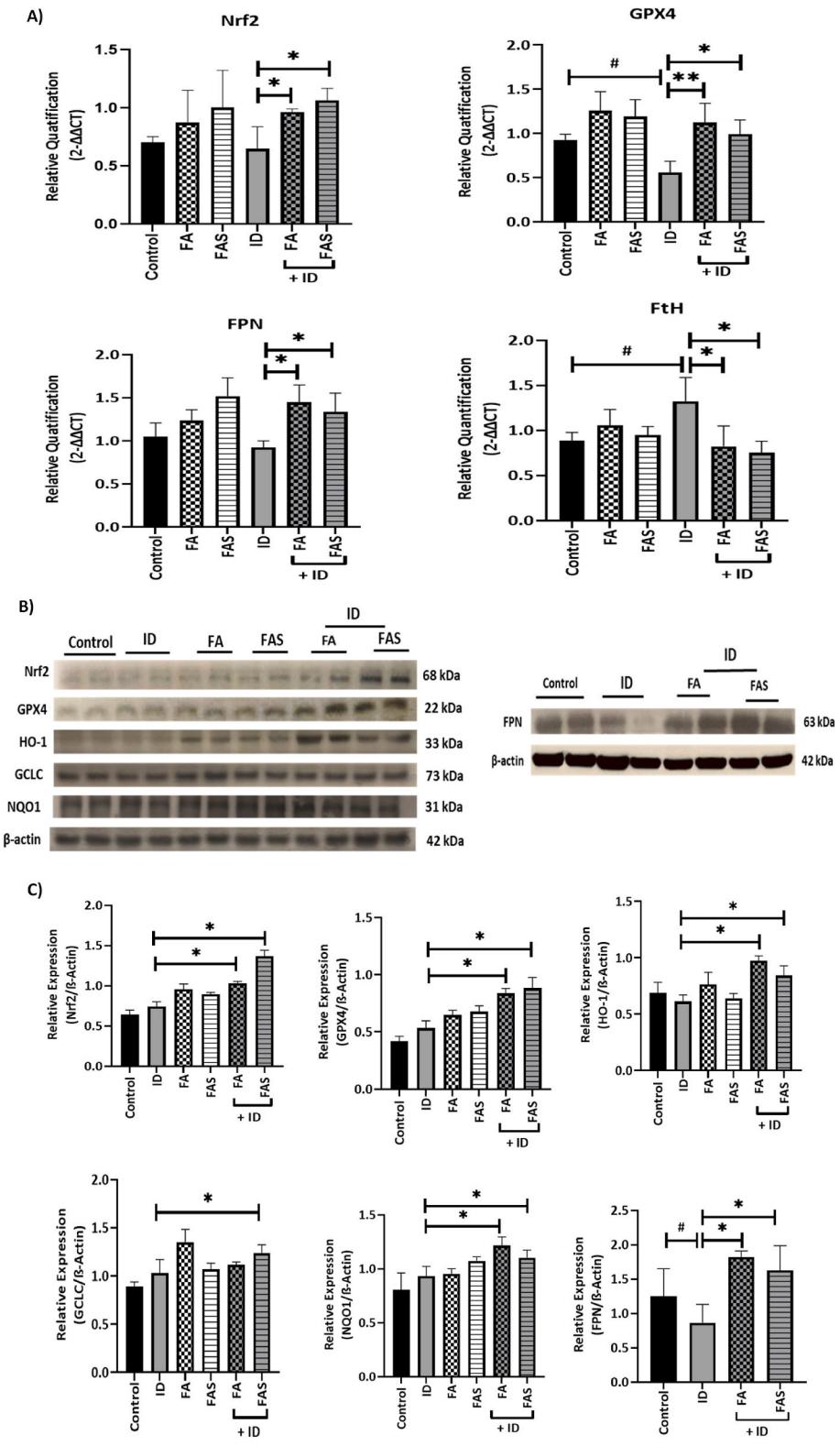

Fig. 5. Protein and gene expressions on effects of phenolic acids in Nrf2 antioxidant pathways. The mRNA levels of Nrf2, GPX4 and FPN were estimated by gene expression by qPCR $2^{-\Delta\Delta CT}$ in the liver and normalized with which housekeeping gene (A). Total proteins were extracted from liver tissues and immunoblot analyses for Nrf2, GPX4, HO-1, GCLC, NQO1 were performed in liver tissues of BALB/c mice (B and C). Antioxidant protein expressions in the liver tissues of only ID treatment were significantly different from the treatment groups of phenolic acids administrated with ID. Densitometry analysis of the blots was analysed with ImageJ software. β-actin served as an internal control. Data are presented as the mean ± SEM, n=5 mice per group. $^{\#}p<0.05$ control group vs. treatment groups, $^{*}p<0.05$, $^{**}p<0.01$ vs. ID group only. One-way ANOVA, Tukey post-hoc test.

3.6. The impact of phenolic acids on the mRNA expression of STAT3, SOCS3 and FoxO1

Furthermore, expressions of mRNA of the inflammation biomarkers were measured in the liver. Fig. 6 shows the relative quantification of signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signalling 3 (SOCS3) and Forkhead box protein O1 (FoxO1). ID treatment groups significantly ($p<0.05$) increased the gene expression of stress-related genes; STAT3, SOCS3 and FoxO1. FA treatment groups exposed to the ID significantly ($p<0.05$) inhibited the mRNA gene expressions STAT3, SOCS3 and FoxO1. However, FAS did not show any significant effect on those genes.

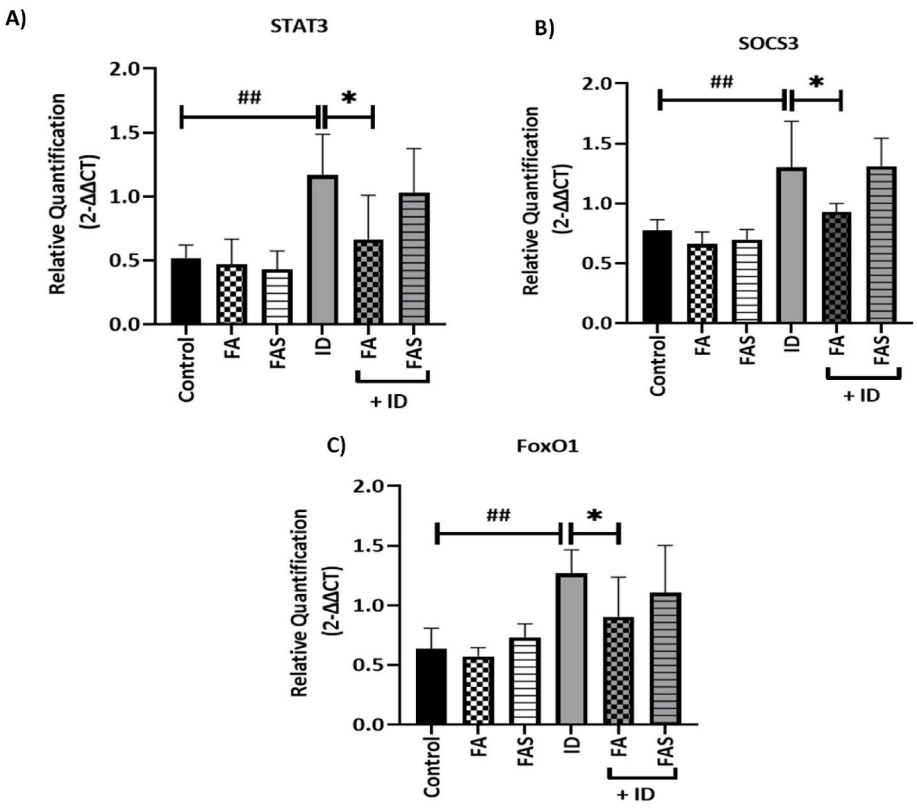

Fig. 6. mRNA expression of STAT3, SOCS3 and FoxO1 genes in the liver tissues. The mRNA levels of signal transducer and activator of transcription 3 (STAT3) (A), suppressor of cytokine signaling 3 (SOCS3) (B), forkhead box O1 (FoxO1) (C) were semi-quantified by RT-PCR analyses in the liver tissues of BALB/c male mice. Administrations of phenolic acids in mice exposed to ID a partially alleviated the impaired mRNA expressions of STAT3, SOCS3 and FoxO1 compared with only ID group. Determination of gene expression by qPCR $2^{-\Delta\Delta CT}$ in the liver and normalized with which housekeeping gene. Data are presented as the mean ± SEM, n=5 mice per group. $^{\#}p<0.05$, $^{\#\#}p<0.01$ Control vs. treatment groups, $^{*}p<0.05$ ID group only vs. treatments groups. One-way ANOVA, Tukey post-hoc test.

4. Discussion

Imbalance of ROS generation due to excess iron accumulation and low antioxidant status offset redox equilibrium thereby leading to oxidative stress, which is a major cause of various tissue injuries [23]. Therefore, the regulation of oxidative stress in tissues is an important strategy to prevent a variety of diseases. Epidemiological, nutritional evidence allude to the beneficial function of natural phenolic compounds in reducing the risk of succumbing to various metabolic and degenerative diseases [24]. FA, a natural phenolic compound, has been reported to exhibit antioxidant, radical scavenging, and metal-chelating effects [25]. FA is predominantly converted into sulfate derivatives during metabolism, an abundant plasma metabolite [26]. Therefore, the current study evaluated the attenuating function of FA and its metabolite, FAS against iron-induced liver organ dysfunctions and HepG2 cell damage. The protection against iron-induced liver tissue injuries and cellular dysfunction by FA was mediated via the Nrf2 antioxidant pathway. The liver is the site for in the detoxification of compounds as well as an iron-storage organ. Iron-induced liver damage and the antioxidant activities of natural products in the liver could be complex to investigate in vivo models [27]. Consequently, HepG2 hepatoma cells were chosen to determine the protective function of FA against iron-induced oxidative stress. The current study revealed the protective activities of FA in HepG2 cells treated with iron and increased levels of SOD and GSH antioxidant enzymes.

The antioxidant functions of phenolic compounds are enhanced by their ability to permeate cell membranes due to their lipophilic structure to prevent the peroxidation of membrane lipids [28]. Similarly, HepG2 cells with 5 μM lipophilic caffeic acid significantly decreased intracellular ROS that was generation induced by 0.3 mM *tert*-Butyl hydroperoxide [29]. Moreover, previous studies have demonstrated that phenolic compounds, beyond their antioxidative activity, can bind metals such as iron and copper [30]. Consequently, curcumin ameliorated iron-induced oxidative stress in AML-12 mouse hepatocytes after exposure to 50 μM FAC [14]. Similarly, a recent in vivo study showed that gallic acid treatment decreased the liver iron content in male C57BL/6J mice with alcoholic liver diseases, thus leading to significantly lower MDA levels compared with the healthy mice group. Additionally, liver GSH and SOD levels were reversed by gallic acid treatment in mice with alcoholic liver diseases [31].

In the current study, protective effects of phenolic acids against iron overload were evaluated in in vivo [32]. This study revealed that significant accumulation of iron in mice liver tissues following iron treatment (Fig. 3). However, supplementations of phenolic compounds, FA, or its metabolite FAS, promoted a decrease in the level of non-heme iron in tissues, suggesting an interaction with iron metabolism in the tissues. FA with OH group on its aromatic group binds iron by chelation scavenges reactive oxygen species and the resultant consequences [8]. Similarly, excess iron accumulated in the liver after ID treatment in Swiss albino mice whereas tannic acid supplementation decreased liver iron levels in the ID group [33]. Iron-chelating natural compounds such as phenolic acids can bind iron tightly to inhibit its reactivity in redox reactions. Furthermore, increased FPN mRNA expression by FA or FAS treatments possibly enhanced iron efflux that culminated in lowered intracellular iron levels in the tissues (Fig. 5). To determine if the natural products-mediated changes in Nrf2 regulated genes were associated with liver iron status, the mRNA levels of the iron storage protein FtH were measured (Fig. 5A). Any changes in tissue iron content are strongly correlated with the expression of FtH, and in the current study, FtH was increased by ID supplementation, in parallel with the notion that iron overload treatment increases liver iron levels by enhancing FtH mRNA levels [34]. When mice were co-treated with FA there was a significant decrease in FtH expression.

ID supplementation caused a significant elevation of hepatic MDA levels in the liver as compared with the control group. The effect, was, however, reversed by the administration of FA or FAS arising from which iron-induced peroxidative damage in the liver decreased. Moreover, both GSH and SOD levels increased in the liver tissues of ID mice treated with FA or FAS compared with ID only treatment group.

Excessive ROS can dysregulate the metabolism of glucose and lipids

in the liver to hasten advancement of hepatic insulin resistance, T2D and NAFLD [35]. The activation of STAT3-SOCS3 pathway by ROS stimulates hepatic insulin resistance. STAT3 is subsequently phosphorylated to induce SOCS3 expression by an inhibitory feedback mechanism. Hence, SOCS3 derange insulin signalling via the ubiquitination through ubiquitin-mediated degradation of insulin receptor substrate 1 [36]. In the current study, FA treatment mitigated the STAT3-SOCS3 signalling in the iron-overloaded group which can indirectly induce insulin resistance (Fig. 6). In parallel, a study by Khodarahmi et al. showed that quercetin administration was associated with a reduction in mRNA from STAT3 and SOCS3 in the liver of oxidative stressed-model mice [36]. Under physiological conditions insulin inactivates FoxO1 by phosphorylation-dependent proteins, demonstrating that FA can mitigate iron-induced insulin resistance by activating insulin signalling [37]. This was evident in the enhancement of nuclear FoxO1 protein level in HepG2 cells treated with 500 μM palmitic acid. This effect was, however, reversed by exposing the cells to 7.5 μM paeonol, a phenolic compound [38].

Dietary phenolic compounds exhibit antioxidant function by ROS scavenging and also by activating Nrf2 antioxidant gene regulation. Antioxidant therapy is a promising therapeutic strategy to prevent or even reverse the progression of oxidative stress by activating Nrf2 pathway. Several natural products, inhibiting the production of ROS, increasing Nrf2 nuclear transcription and activating antioxidant gene expressions, ultimately maintain the physiological balance of the body [39]. Although several natural compounds could induce Nrf2 signalling antioxidant pathway, the precise mechanism by which these compounds influence the Nrf2 pathway is poorly understood [39,40]. For instance, caffeic acid, a phenolic compound, can be used as an electrophile in addition to its nucleophilic nature, which can provide Nrf2 transcription [40]. In the study by Pang et al., it was demonstrated that caffeic acid increased the Nrf2 gene expressions, thereby activating Nrf2 and leading to increased expression of antioxidant signals and reduced cellular ROS levels including HO-1 and NQO1 to prevent acetaminophen-induced acute liver failure in human healthy liver L-02 cells and HepG2 cells [41]. Also, FA is a rich monomer ingredient with a methoxy group on the benzene ring, which makes it a strong ROS scavenger [7]. In the study by Catino et al., FA regulated the Nrf2/HO-1 system and counteracted trimethyltin-induced neuronal disorder in the human neuroblastoma cell lines SH-SY5Y [42]. In addition, FA abrogated oxidative stress and DNA damage by up-regulating Nrf2 gene levels in Swiss albino mice exposed to γ-radiation [43]. In parallel with these results, this study revealed that FA treatment activated the Nrf2 upregulation under the ID supplementation in the liver of BALB/c mice, diminished the oxidative stress markers.

The FA dose used (20 mg/kg) in the current study was calculated according to Reagan-Shaw et al. [44]. The human equivalent dose for the mice is 1.6 mg/kg, which corresponds to around 112 mg of FA consumed daily by human adults. The daily recommended FA supplement dose is 100–500 mg, which is also in accordance with the FA dose applied in the current study [45]. FA is absorbed more quickly and is more bioavailable than other phenolic compounds [46]. A limitation of the current study is that FA and its metabolites were not quantified in the plasma or urine samples of the experimental mice. FAS is a dominant circulating metabolite of FA [47]; however, there is little information regarding its biological activity. Sulfation of phenolic compounds at the active reducing hydroxyl sites inhibits their antioxidant functionality [48]. However, a study by Van Rymenant et al. in Swiss mice showed that FAS exhibited enhanced bioactivity, increasing arterial relaxation, and lowering blood pressure to a greater extent than the native FA compound [49]. Our data confirm that FAS is a bioactive compound that can mitigate oxidative damage in HepG2 cells and in iron-loaded tissues.

A limitation of the current work is the lack of histopathology staining of liver tissues and iron localization. However, we have included direct quantitative measurements of iron content (Fig. 3) and ferritin H (Fig. 5). Both are reduced in iron-loaded mice following FA treatment. These findings justify the assertion that FA modulates cellular and tissue iron metabolism.

In conclusion, we have shown that both FA and FAS are equally effective in limiting iron-induced oxidative stress. Both phenolic acids function through iron chelation and induction of the Nrf2 signalling pathway. The current study has implications for the management of oxidative damage in iron overloaded tissues. This possibility requires further investigation, including a longer-term study of FA supplementation in other disorders that are associated with oxidative stress to confirm the efficacy of this approach.

Author contributions

Conceptualization, GOL-D and TK.; methodology, TK, JMF., M.V.-A. writing—original draft preparation, TK. and—review and editing, GOL-D., and PAS. and M.S.; supervision, GOL-D., and PAS. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding. Tugba Kose received financial sponsorship and a scholarship from the Turkish Government Ministry of National Education.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tugba Kose reports administrative support and equipment, drugs, or supplies were provided by King's College London.

Data availability

The authors do not have permission to share data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101521.

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