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Antioxidative Effects of Curcumin on Erastin-Induced Ferroptosis Through GPX4 Signalling

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Abstract: Background/Objectives: Pancreatic cancer is a common gastrointestinal cancer with high risk of mortality. Currently, the therapeutic strategies for pancreatic cancers are surgery, chemotherapy, and radiotherapy, none of which are effective treatments. Ferroptosis is a new form of cell death that is iron (Fe)-dependent and characterized by lipid peroxidation, which is a new approach for treatment of pancreatic cancer. Therefore, this study was dedicated to investigating the effect of erastin and Ras-selective lethal small molecule 3 (RLS3) as ferroptosis inducers as well as focusing on the antioxidant effects of two natural products, curcumin and (−)-epigallocatechin-3-gallate (EGCG), against ferroptosis. **Methods**: PANC1 cells were treated with 20 µmol/L curcumin or EGCG and then exposed to 20 µmol/L erastin. Cell viability was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay, Reactive Oxygen Species (ROS) were measured by dihydrodichlorofluorescein (H2DCF) cell-permeant probe, Fe levels were determined by inductively coupled plasma mass spectrometry (ICP-MS), and glutathione (GSH), lipid peroxidation, Western blot, and mRNA were assayed with commercially available kits. **Results**: Curcumin and EGCG enhanced cell viability in erastin-treated PANC1 cells in a dose-and time-dependent manner. Erastin-treated PANC1 cells exhibited the elevated levels of GSH depletion, ROS productions, and lipid peroxidation while curcumin reversed the erastin-induced ferroptotic effects. The treatment of erastin-induced PANC1 cells with curcumin increased the GPX4 mRNA gene and protein levels. Also, curcumin decreased the FTH1 mRNA gene levels as a strong Fe chelator. **Conclusions**: In conclusion, this study shows that erastin can be potentially a therapeutic strategy for treatment of cancer cells. Additionally, curcumin might play an antioxidant role at the specific concentrations, potentially mitigating ferroptosis in cells.

Keywords: ferroptosis; curcumin; (−)-epigallocatechin-3-gallate (EGCG); antioxidant effect

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1. Introduction

Pancreatic cancer is a highly aggressive condition of the gastrointestinal system with a survival rate of under 10% over five years [1]. However, there are several challenges in treating pancreatic cancer, including resistance to chemotherapy and radiotherapy, as well as a limited availability of chemotherapy drugs [2]. Therefore, it is essential to develop and design therapeutic strategies with more effective and low drug resistance for treatment of pancreatic cancer patients.

Increasing pathological evidence suggested that iron (Fe) plays a crucial role in initiating and mediating regulated cell death by triggering oxidative damage [3]. Notably, ferroptosis is a newly identified Fe-dependent form of regulated cell death in cancer cells; interestingly, the accumulation of other metals cannot induce ferroptosis [4]. Excess Fe leads to ferroptosis due to production of reactive oxygen species (ROS) via the Fenton reaction, which eventually builds up hydroxyl radicals from hydrogen peroxide or superoxide [5]. In addition, increase in one lipid peroxidative product, malondialdehyde (MDA), is an indicator of ferroptotic cell death [6]. Several molecules have recently been identified to regulate ferroptosis by specific pathways of lipid peroxidation or Fe metabolism. Among them, glutathione peroxidase 4 (GPX4) exhibits strong anti-ferroptosis activity by inhibition of Fe-induced lipid peroxidation. Therefore, inhibition of GPX4 expression or activity induces ferroptosis [7]. Taken together, ferroptosis can be identified, which differs from general necrosis, autophagy, and apoptosis in that it involves an accumulation of both cellular Fe and ROS, and the disappearance of mitochondrial ridges [8]. Even though the physiological function of ferroptosis is obscure, it has drawn interest for its potential therapeutic value in oncological treatment. Ferroptosis inducers can be categorized into two main types. The first type, including erastin, acts by inhibiting the cystine-glutamate transporter (System Xc^-) and disrupting glutathione synthesis. The second type directly targets glutathione peroxidase (GPX) activity, with RSL3 being a key example [6,9]. Ferroptosis inducers, erastin and RSL3, have exhibited anticancer effects in hepato-cellular carcinoma cells, which decreased GSH levels. Anticancer effects of ferroptosis inducers on ovarian, pancreatic, and renal cancers have also been reported [10,11].

It is crucial to develop both highly active anticancer strategies and drugs with minimal side effects. Curcumin is a promising pharmaceutic agent with several therapeutic properties, including antitumor, antimicrobial, and antioxidant activities [12]. Similarly, (−)-epigallocatechin-3-gallate (EGCG) is a major catechin compound in green tea, which plays a key role in regulating reactive oxygen species (ROS) production [13]. Both curcumin [14] and EGCG [12] have garnered attention for their potential to protect against ferroptosis and enhance chemotherapeutic treatments. However, the prooxidant and antioxidant effects of these compounds are concentration dependent.

In this study, we aimed to show whether PANC1 cancer cells are sensitive to ferroptosis inducers. Furthermore, we sought to determine the antioxidant concentrations of two natural compounds, curcumin and EGCG, as inhibitors of ferroptosis activities in PANC1 cells. These findings validate the novel pharmacological activities of curcumin in the protection against cellular ferroptosis.

2. Results

2.1. Effect of Ferroptosis Inducers in PANC1 Cells

To investigate the effects of these two ferroptosis inducers, PANC1 cells were treated with erastin and RSL3 for 24 h (Figure 1). Both compounds reduced cell viability in a dose-dependent manner. As shown in Figure 1, erastin exhibited a more pronounced toxic effect compared to RSL3. Based on these findings, 20 μmol/L erastin exhibited cytotoxic activity at low concentrations; thus, this amount was selected as the ferroptosis inducer for subsequent experiments.

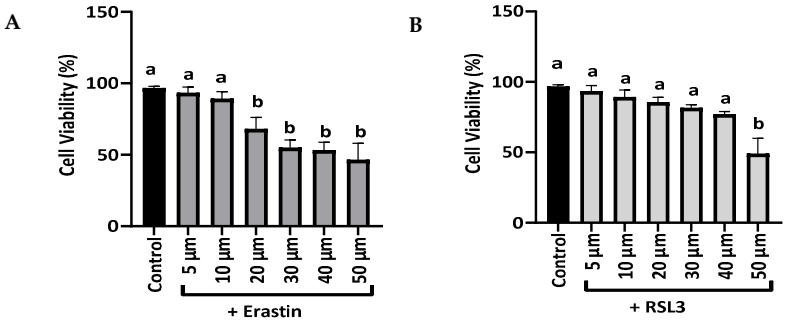

Figure 1. Determination toxic effects of erastin and RSL3. PANC1 cells were exposed to different doses of erastin (**A**) and RSL3 (**B**) for 24 h to assess the toxic effects of ferroptosis inducers. Data are means ± SEM, $n = 8$, independent measurements. Groups not sharing common letters are statistically different ($p < 0.05$), one-way ANOVA and Tukey's post-hoc test.

2.2. Erastin Induces Ferroptotic Cell Death in PANC1 Cells

To assess the protective effects of EGCG and curcumin on erastin-induced ferroptosis in PANC1 cells, the cells were treated with or without EGCG and curcumin (5, 10, and 20 μmol/L) in the presence of 20 μmol/L erastin for 24 h. The reduction in cell viability caused by erastin was fully rescued by treatment with 20 μmol/L EGCG or curcumin (Figure 2A). Thus, 20 μM EGCG or curcumin were used as a treatment model in the experiments. Both EGCG and curcumin inhibited erastin-induced cell death in a dose-dependent (Figure 2C) and time-dependent (Figure 2D) manner. Additionally, when PANC1 cells were treated with varying concentrations of the ferroptosis inhibitor Fer-1 alongside 20 μmol/L erastin, 5 μmol/L Fer-1 effectively prevented erastin-induced cell death (Figure 2A).

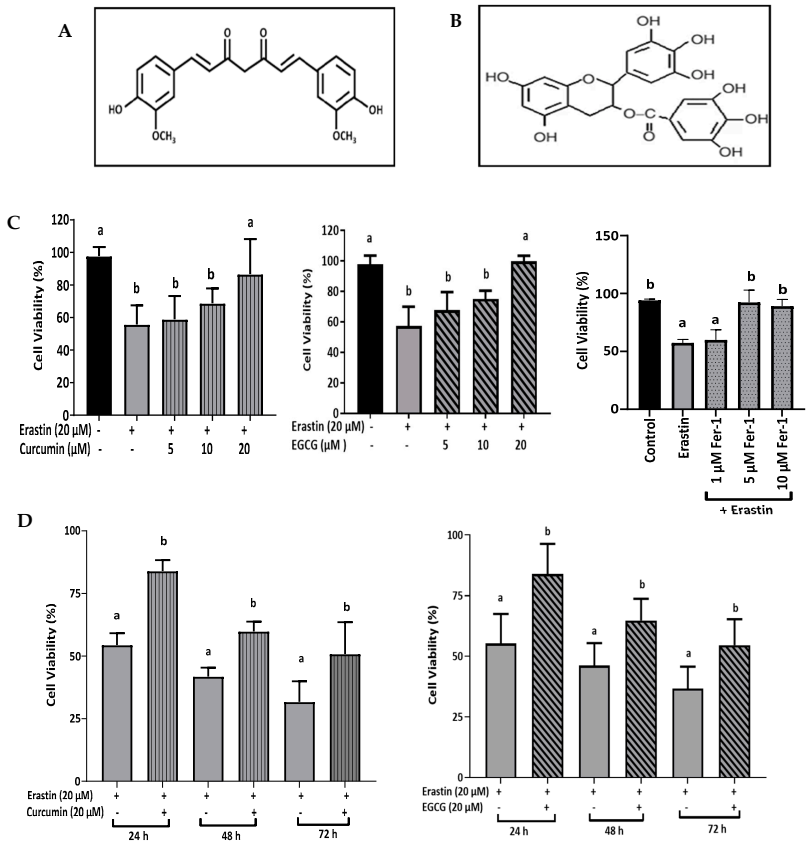

Figure 2. Anti−ferroptosis effects of curcumin and EGCG in PANC1 cells. Chemical structure of curcumin (**A**) and EGCG (**B**). PANC1 cells were supplemented with 20 μmol/L erastin for 24 h in

the absence or presence of different doses of curcumin or EGCG or Fer-1. Curcumin and EGCG inhibited erastin-induced cell death in a dose- (**C**) and time- (**D**) dependent manner. Fer-1 indicated inhibitor effect against ferroptosis for 24 h. The percentage of cell viability is relative to control cell samples. Data are means ± SEM, n = 8, independent measurements. Groups not sharing common letters are statistically different ($p < 0.05$), one-way ANOVA and Tukey's post-hoc test.

2.3. Curcumin Decreased Iron Accumulation, Lipid Peroxidation and ROS

Accumulation of both ROS-dependent lipid peroxidation products and Fe contribute to erastin-induced ferroptosis. The protective functions of curcumin and EGCG were therefore investigated in PANC1 cells (Figure 3). The end product of lipid peroxidation, MDA, which is the significant biomarker of lipid peroxidation in ferroptosis, was dramatically increased following treatment with erastin in PANC1 cells (Figure 3B). However, curcumin effectively decreased the erastin-induced MDA production. Also, curcumin decreased erastin-induced Fe accumulation (Figure 3C). To confirm the Fe accumulation in the cells, FTH1 mRNA levels were significantly increased in erastin treated PANC1 cells (Figure 3D).

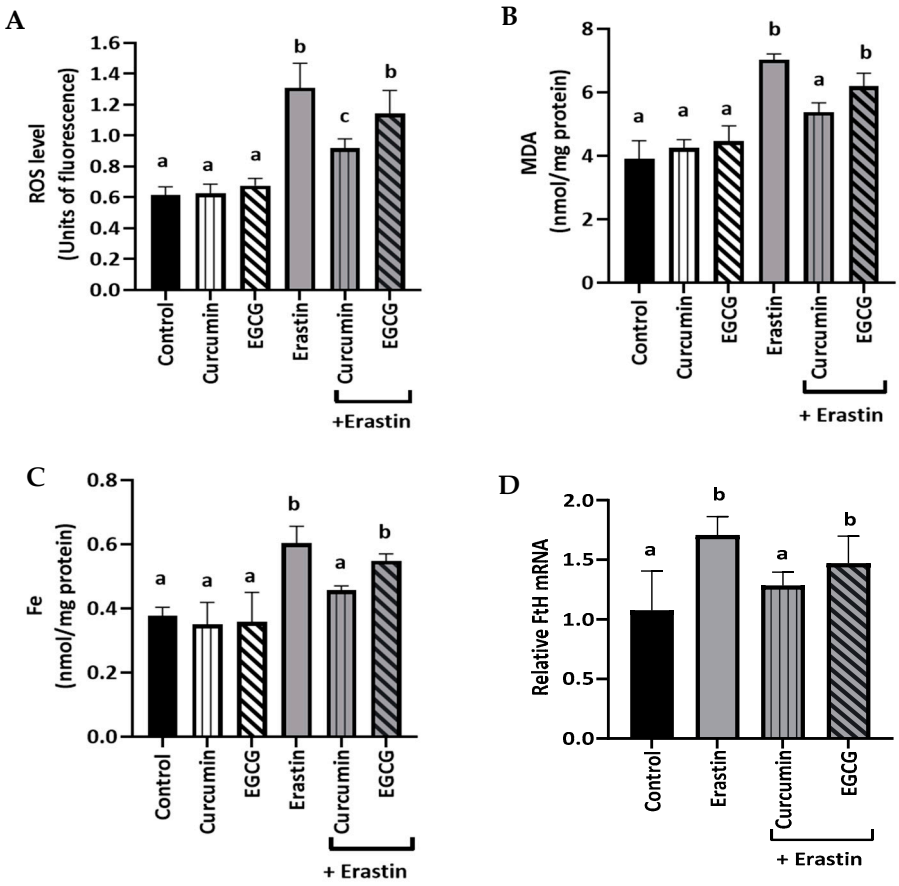

Figure 3. Effects of curcumin and EGCG on erastin-induced iron overloading, lipid peroxidation, and intracellular ROS production. PANC1 cells were treated with 20 μmol/L of erastin in the absence and presence of 20 μmol/L of curcumin or EGCG for 24 h. Then, Fe concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS) (**A**), The mRNA level FTH1 were analysed by qPCR (**B**), lipid peroxidation levels were determined by malondialdehyde (MDA) assay (**C**), cellular reactive oxygen species (ROS) were monitored with a dihydrodichlorofluorescein (H2DCF) cell-permeant probe (**D**). Data are means ± SEM, n = 3, independent measurements. Groups not sharing common letters are statistically different ($p < 0.05$), one-way ANOVA and Tukey's post-hoc test.

2.4. Curcumin Could Suppress GSH Depletion

GSH depletion is a key factor in the initiation of lipid peroxidation during ferroptosis, as GSH is an essential cofactor for the antioxidant enzyme GPX4 [7]. Treatment with erastin exacerbated GSH depletion in PANC1 cells (Figure 4A). However, curcumin mitigated the erastin-induced GSH depletion (Figure 4A) and protected against the degradation of GPX4 mRNA levels in PANC1 cells (Figure 4B). Consistent with the results of GSH and GPX4 mRNA levels, GPX4 protein levels were significantly elevated in curcumin treated PANC1 cells exposed to erastin (Figure 4C).

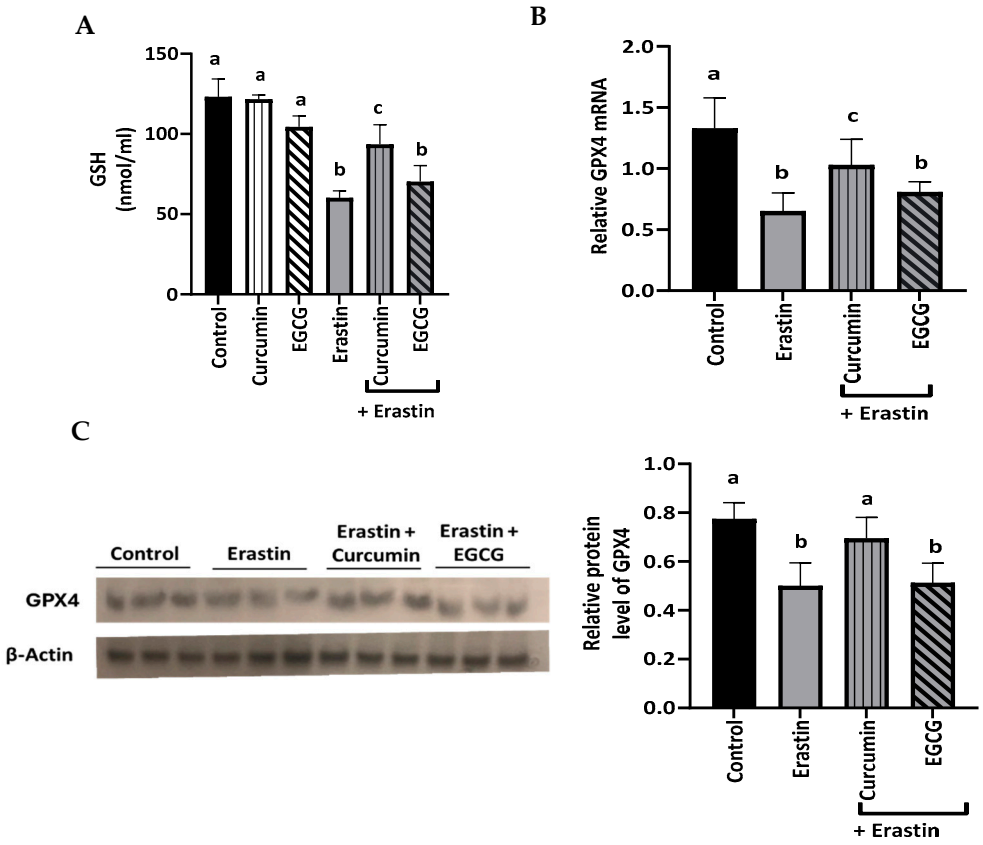

Figure 4. Anti-ferroptosis activity of curcumin in PANC1 cells against erastin-induced GSH depletion and GPX4 inactivity. PANC1 exocrine cells were treated with erastin (20 μmol/L) in the absence or presence of curcumin or EGCG (20 μmol/L) for 24 h. GSH levels (**A**) and indicated protein levels (**B**) and western blot (**C**), were determined by assay respectively. Data are means ± SEM, n = 3, independent measurements. Groups not sharing common letters are statistically different ($p < 0.05$), one-way ANOVA and Tukey's post-hoc test.

3. Materials and Methods

The antibodies to GPX4 (67763-1-Ig) and β-Actin (20536-1-AP) were obtained from Thermo Fisher Scientific (Loughborough, UK). Erastin, curcumin, EGCG, and ferrostatin-1 (Fer-1) were purchased from Sigma Aldrich (Dorset, UK). All other reagents were procured from Sigma Aldrich (Dorset, UK) unless specified.

3.1. Cell Culture

PANC1 cell line (human pancreatic ductal adenocarcinoma cells) was purchased from the American Type Culture Collection. This cell line was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Invitrogen, Waltham, MA, USA), 0.1 mg/mL streptomycin, and 100 U/mL penicillin (Invitrogen). PANC1 cells were kept at 37 °C under a humidified atmosphere with 5% CO_2. This cell line (<30 passages)

was cultured in T75-cm^2 plastic flasks. The medium was changed twice a week. PANC1 cells were used for splitting when 80–90% confluent. In experiments, the PANC1 cell line was exposed to erastin with/without polyphenols for 24 h. Control PANC1 cell groups were treated with 0.01% dimethyl sulfoxide (DMSO).

3.2. Cell Viability Assay

Effects of curcumin and EGCG against erastin and RSL3-induced cell death were assessed in PANC1 cells. PANC1 cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. PANC1 cells were seeded at a density of 10×10^4 cells per well in a 96-well plate and cotreated with curcumin and EGCG, as well as being exposed to erastin or RSL3 at varying concentrations for 24 h. The control group was treated with 0.01% DMSO, which was used to normalize the effects of the treatment groups. In total, 10 µL formazan products of MTT were dissolved in 100 µL of fresh DMSO and incubated for 15 min at room temperature, measured as absorbance at 570 nm in a spectrophotometer. Cell viability was expressed as a percentage of the controls [15].

3.3. Cellular Iron Levels

To measure total cellular Fe level in PANC1 cells, inductively coupled plasma mass spectrometry (ICP-MS) analysis was performed. PANC1 cell pellets were collected for metal analysis by ICP-MS, then were dissolved in 200 µL of 50 mM NaOH. The samples were supplemented with concentrated 68% nitric acid (HNO_3), then heated for 3 h at 80 °C to complete the cell digestion. For the Fe measurement, the Agilent ICPMS 7700 x series ICPMS instrument (Agilent Technologies, Waldbronn, Germany) was used under suitable conditions for routine multi-element analysis.

3.4. Lipid Peroxidation Assay

To measure the MDA amounts (one of the end products of lipid peroxidation) in PANC1 cells, a lipid peroxidation assay kit was purchased from Cohesion Biosciences (London, UK) and used according to the manufacturer's instructions. The absorbance of the supernatant of PANC1 cells was assessed at 532 and 600 nm. Cellular MDA levels were normalized with protein content and expressed as nmol/mg protein.

3.5. Reactive Oxygen Species (ROS)

To determine ROS amount in PANC1 cells, a Dihydrodichlorofluorescein (H2DCF) cell-permeant probe was used according to the manufacturer's recommendations. PANC1 cells from different treatment groups were collected and washed with fresh PBS and then incubated during 90 min in the dark at 37 °C in PBS including 10 µmol/L of H2DCF. The level of ROS in PANC1 cells in the different treatment groups was determined by flow cytometry based on the fluorescence intensity of DCF at 525 nm after excitation at 485 nm.

3.6. Glutathione Assay

The glutathione (GSH) concentration in PANC1 cell lysates was assessed by using a glutathione assay kit purchased from Sigma Aldrich (Dorset, UK) according to the manufacturer's instructions. The absorbance of the mixtures was read at 412 nm at 1-min intervals 5 times. The level of GSH was indicated as nmol/mL.

3.7. Western Blot

The total proteins of PANC1 cells were extracted using fresh, cold RIPA buffer. Both a protease inhibitor and the protein concentrations were detected by BCA protein assay kits (Thermo Fisher Scientific, Waltham, MA, USA). Then, 15 µg protein of each sample was

subjected to SDS-PAGE gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% non-fat dry milk and incubated with primary antibodies (GPX4 and GADPH) overnight at 4 °C. Then, the membranes were incubated with HRP-conjugated secondary antibody (diluted 1:5000, R&D Systems, Abingdon, UK) for 1 h at room temperature. Images were visualized by Clarity Western ECL Substrate (Watford, UK) and integrated density was quantitated via ImageJ software (v1.51w) (Adobe Systems, San Jose, CA, USA).

3.8. RNA Extraction and Real-Time PCR

The total RNA in PANC1 cells was extracted by Trizol reagent according to the manufacturer's instructions. cDNA synthesis was performed via cDNA Synthesis SuperMix and SYBR Green Master Mix and ABI 7500 were used to assess the mRNA expression levels of FTH1 and GPX4 by qPCR. GAPDH, an internal reference and relative target gene expression, was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were listed in Table 1.

Table 1. Primers for qPCR analysis.

Gene Name	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)
GPX4 (NM_002085.5)	GAGGCAAGACCGAAGTAAACTAC	CCGAACTGGTTACACGGGAA
FTH1 (NM_002032.3)	TTCAACAGTGCTTGGACGGA	ATCACTGTCTCCCAGGGTGT
GAPDH (NM_002046.3)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

3.9. Statistical Analysis

Data were expressed as means ± SEM. Statistical analysis was performed by GraphPad Prism 10.0 (GraphPad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-hoc test to compare the means of the experimental groups. Data represent duplicate measurements from three independent experiments. Groups that do not share the same letters are significantly different ($p < 0.05$).

4. Discussion and Conclusion

Pancreatic cancer is a highly aggressive form of gastrointestinal disease and can progress rapidly [16,17]. This study aimed to identify the optimal dose of erastin as a ferroptosis inducer and the appropriate concentrations of curcumin or EGCG as ferroptosis inhibitors. The anti-ferroptosis activities of curcumin were involved in the modification of Fe accumulation, ROS production, and lipid peroxidation in erastin-treated PANC1 cells. Furthermore, curcumin prevented erastin-induced GSH depletion by up-regulating GPX4 gene and protein levels, which alleviate lipid peroxidation at the dose-dependent manner.

Pancreatic cancer is highly resistant to radiotherapy, chemotherapy, and other drug treatments [18]. Given that several agents that induce ferroptosis, such as erastin, RSL3, or sulfasalazine, are already US Food and Drug Administration-approved, ferroptosis offers a crucial therapeutic approach for cancers that are particularly difficult to treat with traditional chemotherapy and have some of the lowest survival rates among cancer types [10]. This study highlights the potential of ferroptosis as an effective strategy to eliminate PANC1 cells, demonstrating that four key mechanisms can effectively trigger ferroptosis in this cell line: GSH depletion, GPX4 inhibition, elevated levels of unstable Fe, and increased ROS production [6].

In the present study, PANC1 exocrine cells were used as an in vitro model as they have both a poor differentiation ability and a particular resistance to chemotherapeutic activity [19]. To demonstrate the efficacy of ferroptosis inducers in PANC1 cell line, cells were treated with the varying concentrations of erastin or RSL3. Among these, 20 μmol/L

erastin indicated high potent ferroptotic activity in PANC1 cells compared to RSL3. Dixon et al. have previously reported that erastin is a fast-acting and more effective ferroptosis inducer than other agents across five different cancer cell lines (BJeHLT, 143B, Calu-1, BJeLR, and HT-1080), demonstrating effectiveness at lower concentrations and producing long-lasting impacts [20]. To show the erastin-induced ferroptotic damage, the PANC1 cell model subjected to 20 μmol/L erastin was used. This similar approach has been implemented in the different cancerogenic cell models, showing that ferroptosis can be used as an effective treatment [21]. Furthermore, natural products can be considered promising compounds for the alleviation of chronic conditions in vivo and in vitro studies at the dose-response effect [22]; thus, EGCG and curcumin were included in the present study to show anti-ferroptotic activity in PANC1 cells. This aligns with a study by Xie et al., which showed that 20 μmol/L baicalein significantly reduced ferroptotic damage in PANC1 cells [23], which can support the potential of natural compounds in modulating cellular ferroptosis.

Curcumin, a prominent polyphenol derived from turmeric, significantly influences the proteins involved in Fe metabolism in both cells and tissues, indicating its potential as an Fe chelator based on its concentration [24]. This Fe-chelating activity of curcumin is attributed to its β-diketone group in its chemical structure. Additionally, curcumin's lipophilic nature allows it to easily cross cell membranes, potentially enabling it to chelate metal ions within cells [25]. On the other hand, EGCG, the primary flavonoid in green tea, has gained considerable pharmacological attention for its anti-inflammatory, antioxidant, and anticancer properties at specific doses [26]. EGCG is highly hydrophilic due to its hydroxyl groups, which allows it to bind ROS and chelate metal ions, including Fe [27]. While the chelation of metal ions by EGCG plays a minor role in its antioxidant action [28], its main effect is attributed to its ability to scavenge free radicals [27]. Interestingly, both curcumin or EGCG could be identified as potential ferroptosis inhibitors, depending on their treatment time and concentration.

Ferroptosis is caused by disruptions in Fe homeostasis and lipid peroxidation pathways. In ferroptotic cells, the accumulation of excess Fe causes the production of ROS, which primarily impair lipid structures in the cell membrane [29]. Thus, the present study hypothesized that erastin treatment could alter the regulators of Fe metabolism in PANC1 cells. The results showed that curcumin ameliorated erastin-induced Fe accumulation and ROS production by down-regulating the FTH1 gene expression in PANC1 cells. Additionally, in PANC1 cells treated with erastin, curcumin treatment reduced MDA levels, a marker of lipid peroxidation. These findings are consistent with a study by Zhou et al., which demonstrated that 8 μmol/L curcumin reduced Fe accumulation and MDA production in 3.33 μmol/L erastin-treated chondrocyte cells by upregulating GPX4 protein levels [30]. It is well-established that ROS plays a critical role in the progression of pancreatic cancer [31], and the levels of ROS in cells can be modulated by antioxidant enzymes such as GSH [32]. In this study, the elevated ROS levels in PANC1 cells might be linked to the reduced activity of another antioxidant enzyme, GPX4, as shown in Figure 4.

PANC1 cells were susceptible to erastin, leading to the inhibition of system Xc^-, which decreases intracellular GSH and inactivates the antioxidant GPX4 enzyme, causing increased lipid peroxidation or lipid-ROS products and ultimately resulting in ferroptosis [19]. However, curcumin mitigated GSH depletion by upregulating GPX4 expression in PANC1 cells treated with erastin, significantly reversing the ferroptosis. Supporting this finding, a study by Samarghandian et al. demonstrated that at a specific concentration (30 mg/kg), curcumin treatment alleviated GSH depletion in liver tissues of Wistar albino rats subjected to restraint stress by normalizing both GPX4 mRNA and protein levels [33]. These results suggest that curcumin can play a restorative role in balancing

ROS and antioxidant levels at the specific dose, thereby counteracting erastin-induced ferroptosis in PANC1 cells. However, effects of curcumin and EGCG as antioxidants or prooxidants in cells are influenced by their concentration and treatment duration [34,35]. For instance, Holczer et al. demonstrated that administering low concentrations of EGCG (10 µmol/L to 20 µmol/L) for 24 h slightly increased the viability of HEK293T cells (human kidney epithelial cell line), while 80 µmol/L of EGCG led to a significant decrease in cell viability by about 50% [36]. Additionally, treatment with 50 µmol/L curcumin for 24 h in A293 cells (human embryonic kidney carcinoma cell line) resulted in GSH depletion and increased ROS production [35]. However, further in vivo studies are necessary to better define the concentration of curcumin or EGCG to maximize their antioxidant and prooxidant properties and their potential therapeutic applications across different cell lines or tissues.

The discovery of drugs with low toxicity and high efficacy that not only exhibit anti-tumour activity but also enhance susceptibility provides new insights for cancer patients. This research suggests that the ferroptosis inducer erastin, when used in the treatment of pancreatic tumours, can has a unique mechanism of action and be effective at low toxicity levels, making it a promising candidate for combination with chemotherapy. Furthermore, the study demonstrates that curcumin has anti-ferroptosis activity and Fe-chelating properties at specific dose in PANC1 cells. However, the mechanisms of erastin in cancer treatment and the precise concentrations of curcumin required for its antioxidant or prooxidant effects need to be further explored in clinical trials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/gidisord7010004/s1.

Author Contributions: G.O.L.-D. supervised the work and edited the manuscript. P.A.S. co-supervised the work and co-edited the manuscript. T.K. designed the experiments and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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