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Antiproliferative Effects of Reynoutrin via Modulation of Oxidative Stress, Inflammation, and Apoptosis in Breast (MCF-7) and Prostate Cancer (PC-3) Cells Lines

Sedat GÖKMEN

sgokmen@kastamonu.edu.tr

Kastamonu University

İrfan ÇINAR

Kastamonu University

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Abstract

Background

Reynoutrin, a bioactive flavonoid, is recognized for its biological effects. Despite toxic side effects, cisplatin is widely used to treat malignancies. This study aimed to assess the *in vitro* antiproliferative activity of reynoutrin in MCF-7 and PC-3 cell lines by evaluating its modulation of intracellular signaling pathways.

Methods

The cytotoxic effects of reynoutrin on MCF-7 and PC-3 cells were evaluated using an MTT assay after 24 h. Oxidative stress levels were measured using enzyme-linked immunosorbent assay (ELISA) with total antioxidant and oxidant status kits. The expression levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, caspase 3, caspase 9, B-cell lymphoma-2 (Bcl-2), and Bcl-2-associated X protein (Bax) were detected by RT-PCR. Apoptosis was verified using Hoechst staining.

Results

Reynoutrin inhibited MCF-7 and PC-3 cell viability in a dose-dependent manner, with IC_{50} values of 220 and 412 μg/mL, respectively. Reynoutrin increased the total oxidant status, decreased the total antioxidant enzyme activity, and enhanced oxidative stress ($p < 0.05$). RT-PCR showed that TNF-α, IL-1β, and IL-6 expression levels decreased in a dose-dependent manner following reynoutrin administration in MCF-7 and PC-3 cells ($p < 0.05$), indicating its anti-inflammatory activity. In cancer cells, reynoutrin increased caspase-3 and caspase-9 levels and decreased the Bcl-2/Bax ratio in a dose-dependent manner ($p < 0.05$).

Conclusion

These findings demonstrate that reynoutrin exerts antiproliferative activity by regulating inflammation, oxidative stress, and apoptotic pathways in cancer cells.

Highlights

Reynoutrin inhibited the viability of MCF-7 and PC-3 cells in a dose-dependent manner.

Reynoutrin increased oxidative stress by elevating TOS and decreasing TAS levels.

Reynoutrin suppressed pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 expression.

Reynoutrin induced apoptosis by upregulating caspase-3, caspase-9, and Bax while downregulating Bcl-2.

Reynoutrin shows promise as a potential therapeutic agent against breast and prostate cancers.

Introduction

Cancer is the second leading cause of death worldwide, represents a major global public health challenge, and is one of the leading contributor to mortality and morbidity. Reports indicate that 19,976,499 new cancer cases and 9,743,832 cancer-related deaths occurred worldwide in 2022. Breast (11.5%) and prostate (7.3%) cancers are among the top five types of cancers with the highest incidence rates worldwide [1].

Antineoplastic agents are fundamental in cancer therapy, often administered alongside radiotherapy, surgery, and immunotherapy [2]. However, their use is frequently limited by significant adverse effects and drug resistance in tumor cells. These drugs can cause mild to life-threatening effects, including gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, hematopoietic system damage, cardiotoxicity, and neurotoxicity [3–7]. Cancer cells frequently acquire resistance to chemotherapeutic agents following treatment. These problems reduce patients' quality of life and drug efficacy, restrict clinical use, and can lead to treatment failure [8, 9]. Therefore, more effective strategies for managing chemotherapy-related side effects are required. Alternative approaches have been investigated to reduce side effects, ensure effective doses, and reduce health care costs. Phytochemicals have been extensively studied for their synergistic effects on chemotherapy regimens and cancer prevention [8–10]. Currently, flavonoids are extensively used in both *in vivo* and *in vitro* studies to explore their pharmacological properties, particularly for cancer treatment. Flavonoids are used to treat a range of diseases including cancer, diabetes, cardiovascular diseases and ulcers. Flavonoids exert cytotoxic effects on cancer cells through multiple mechanisms, including anti-inflammatory activity, induction of apoptosis, and disruption of oxidative stress balance. Among the studies conducted on the anticancer activity of these compounds, hesperidin, quercetin, cyanidin, catechins, apigenin, and quercetin-3-*O*-glucoside are of particular interest [11–13]. Studies that have reached the final phase have demonstrated promising results, indicating that these compounds may serve as effective adjuvant agents in the treatment or reduction of the risk of various types of cancer [14].

Reynoutrin, also known as quercetin-3-D-xyloside, is a flavonoid naturally found in the fruits and leaves of various plants [15]. Although studies of reynoutrin are limited, existing research suggests that it exhibits a wide range of pharmacological activities. These include antitumor, antiviral, and antidiabetic effects [15–17]. Additionally, reynoutrin has been associated with wound healing properties and has potential benefits in improving ischemic heart failure [18].

Oxidative stress, an imbalance between reactive oxygen species (ROS) production and antioxidant defense systems, plays a significant role in carcinogenesis, tumor progression, and chemoresistance. Flavonoids have a dual role in ROS homeostasis; they act as antioxidants under physiological conditions

but as pro-oxidants under pathological conditions, such as cancer, initiating apoptotic pathways. Pro-oxidant activity is a key mechanism underlying the anticancer potential of flavonoids [19]. Flavonoids, including daidzein, hesperidin, and naringenin, exert pro-oxidant effects in cancer cell lines such as MCF-7 and PC-3 by elevating intracellular ROS levels and enhancing oxidative stress. This increase in ROS disrupts cellular redox homeostasis, leading to the suppression of cancer cell proliferation and migration, and the induction of cell death [20].

Inflammation causes tumor development and regulates cellular differentiation, survival, proliferation, invasion, metastasis, and angiogenesis [21]. Inflammation facilitates tumor initiation and progression by promoting proliferative signaling, angiogenesis, and resistance to apoptosis. Proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) contribute to tumorigenesis. Flavonoids (such as apigenin, rutin, and pelargonidin) suppress inflammation by downregulating pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-1β, thereby inhibiting tumor development. IL-6, IL-1β, and TNF-α regulate the functions of various cell types, including breast [22] and prostate [23] cancers, particularly tumor growth, invasion, and metastasis [24].

Apoptosis is a type of programmed cell death that maintains homeostasis and eliminates the damaged cells. The Bcl-2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2) are critical regulators of apoptosis and cell survival, respectively. Bax promotes cell death, whereas Bcl-2 promotes cell survival. A decreased Bcl-2/Bax ratio indicates increased susceptibility to apoptosis [25]. The intrinsic apoptotic pathway is regulated by pro-apoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2, which control mitochondrial membrane permeability and cytochrome *c* release. This activates caspase-9 and caspase-3, leading to DNA fragmentation and cell death [26]. Research has shown that caspase-3 and caspase-9 are essential mediators of flavonoid-induced apoptosis in cancer cells [27]. Caspase-9 initiates the apoptotic cascade, whereas caspase-3 cleaves cellular substrates during apoptosis [28]. Studies have shown that flavonoids induce apoptosis by upregulating pro-apoptotic markers and downregulating Bcl-2 [29].

Currently, studies on reynoutrin are limited, and its antiproliferative activities, particularly in breast and prostate cancers, have not been clarified. In this study, we investigated the antiproliferative potential of reynoutrin by evaluating its effects on oxidative stress parameters (total antioxidant and oxidant status), proinflammatory cytokines (TNF-α, IL-1β, and IL-6), and key apoptotic markers (caspase-3, caspase-9, and Bcl-2/Bax) in MCF-7 and PC-3 cells. These findings provide valuable insights into the molecular mechanisms underlying the anticancer effects of reynoutrin, and support its potential as a complementary cancer therapy.

Materials and methods

Chemicals and reagents

Reynoutrin (Cas number: 549-32-6; purity > 97%) and cisplatin (Cas number: 15663-27-1; purity > 99.84%) were purchased from MedChemExpress (Manmouth Junctions, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), cell culture medium, and reagents such as fetal bovine serum (FBS), penicillin/streptomycin, and trypsin–ethylenediaminetetraacetic acid (trypsin/EDTA) were obtained from Gibco (Invitrogen Inc., Grand Island, New York, USA).

Cell culture procedures

Human breast (MCF-7) and prostate (PC-3) cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines stored in the liquid nitrogen tank were briefly thawed in a 37°C water bath and subsequently transferred into T75 cm^2 flasks for culture. The cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (100 IU/mL) at 37°C in a humidified 5% CO_2 incubator. All incubations conducted on the cells throughout the study were performed in a CO_2 incubator (PHCbi cell culture incubator, Loughborough, UK) at 37°C in a medium containing 5% CO_2. Upon reaching approximately 80% confluence, cells were detached with 0.25% trypsin/EDTA and subsequently seeded into 6-well plates for RNA extraction and biochemical analyses and into 96-well plates for MTT assays. After the cells reached approximately 80% confluence, they were detached with 0.25% trypsin/EDTA and plated for RNA extraction and biochemical investigations in 6-well plates, and for the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide] assay in 96-well plates. All cell culture procedures were performed in compliance with standards described in the literature.

Cell viability assay

The MTT (Thermo Fisher Scientific, Waltham, MA, USA) assay was performed to determine cell viability according to the manufacturer's instructions. MCF-7 and PC-3 cells were cultured in 96-well plates (5 × 10^3 cells/well) and were allowed to attach. The cells were then exposed to different concentrations (10–2000 µg/mL) of reynoutrin, dissolved in dimethyl sulfoxide (DMSO) as the solvent. The final DMSO concentration was maintained at 0.1% in all treatment and control groups, and incubated for 24 h to determine the IC_{50} (half-maximal inhibitory concentration). These concentrations were investigated in previous studies. Following the completion of exposure, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well, and the plate was incubated at 37°C for 4 h. Dark crystals of formazan formed in intact cells were dissolved in 200 µL of DMSO. After shaking the plates for 15 min, the absorbance at 570 nm was measured using a microplate spectrophotometer (Epoch Microplate Spectrophotometer, BioTek, USA) in accordance with the manufacturer's protocol (Roche, Germany). The cell viability rate (%) was calculated using the following equation.

$$\% \text{ Cell viability rate } = \frac{OD_{test\ sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100$$

$OD_{test\ sample}$: Optical density (absorbance) measured in the wells containing the test sample.

OD_{blank}: Absorbance of a blank well (medium without cells) subtracted from the background.

$OD_{control}$: Absorbance of the untreated control well

The IC_{50} concentrations were calculated using Graphpad Probit Analysis Program (Version 9.3.0).

Experimental groups

To investigate the antiproliferative effects of reynoutrin, six experimental groups were established for both the MCF-7 and PC-3 cell lines. The groups were designed as follows:

Group I (Control; C): No chemical treatment was administered to MCF-7 or PC-3 cells.

Group II (DMSO): MCF-7 and PC-3 cells treated with %0.1 DMSO for 24 h.

Group III (Cis50): MCF-7 and PC-3 cells were exposed to 50 μM cisplatin for 24 h.

Group IV (Rey Low Dose): Cells were treated for 24 h with a lower concentration of reynoutrin: 110 μg/mL for MCF-7 cells and 206 μg/mL for PC-3 cells.

Group V (Rey Mid-level Dose): Cells were treated for 24 h with moderate concentrations of reynoutrin, 220 μg/mL for MCF-7 and 412 μg/mL for PC-3.

Group VI (Rey High Dose): Cells were treated for 24 h with a higher concentration of reynoutrin, 330 μg/mL for MCF-7 and 618 μg/mL for PC-3.

Determination of intracellular antioxidant–oxidant status and oxidative stress index

The total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) are oxidative profiles that determine the intracellular oxidative. We measured TAS and TOS using Rel Assay Diagnostics Assay Kits (Rel Assay Diagnostics, Gaziantep, Turkey). The parameters were measured using a spectrophotometric method with an Epoch microplate reader following the manufacturer's instructions. The color change was measured at a wavelength of 660 nm, and the TAS results were expressed as mmol Trolox equivalents per mg of protein, whereas the TOS levels were determined based on the color change measured at 530 nm and expressed as μmol H_2O_2 equivalents per mg of protein. OSI was calculated using the following formula [31]:

$$\mathrm{OSI\ (Arbitrary\ Unit,\ AU)} = \frac{\mathrm{TOS\ (\mu\ mol\ } H_2O_2\ \mathrm{Equiv./mg\ protein)}}{\mathrm{TAS\ (mmol\ Trolox\ Equiv./mg\ protein)}} \times 10$$

Each experiment was conducted in three independent replicates.

Quantitative determination of mRNA expression by RT-PCR

The mRNA expression levels of IL-1β, IL-6, TNF-α, caspase-3, caspase-9, Bax, and Bcl-2 were analyzed using Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The cells were collected from a 6-plate using a scraper and 350 µL of RLT solution was added separately to each group, followed by homogenization using a Tissue Lyser II device (Qiagen, Germany). RNA extraction was performed according to the manufacturer's instructions using an isolation kit (Qiagen RNA, Cat. No: 74,104, Qiagen, Hille, Germany). The RNA concentration was measured using a NanoDrop spectrophotometer (Epoch 2, BioTek, USA). The quality of RNA was determined using optical densities (OD260/280) per nanodrop, and RNAs with OD260/280 ratios between 1.7 and 2.2 (or ~ 1.8) were used in the study. For cDNA synthesis, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) was used for cDNA synthesis. The process was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems), and the synthesized cDNA was preserved at -20°C. The mRNA expression of IL-1β (Hs01555410_m1), IL-6 (Hs00174131_m1), TNF-α (Hs00174128_m1), Caspase-3 (Hs00234387_m1), Caspase-9 (Hs00154260_m1), Bax (Hs00180269_m1), and Bcl-2 (Hs04986394_s1) was measured using the TaqMan Master Mix kit. RT-PCR amplification and quantification were conducted using a StepOnePlus device (Applied Biosystems). Gene Expression levels were normalized to the β-actin reference gene Hs01060665_g1 TaqMan® Gene Expression, which was modified to the PCR program according to the kit's instructions, and the process was initiated. All results are represented as fold-changes in expression compared with the groups using the $2^{-\Delta\Delta Ct}$ method. Each gene was analyzed using the approach described in our previous study [30]. Each experiment was conducted in triplicate.

Hoechst 33342 staining

As characteristic changes in nuclear morphology typically occur during apoptosis, these changes can serve as indicators of programmed cell death. Apoptotic cell death was determined by Hoechst 33342 staining. Hoechst 33342 fluorescence staining was used to evaluate apoptotic cells according to their nuclear morphology. Hoechst 33342, a blue fluorescent membrane-permeable dye, stains condensed chromatin more brightly in apoptotic cells than in normal cells. For this purpose, MCF-7 and PC-3 cells (2×10^5 cells/well) were seeded onto 6-well plates prior to treatment with reynoutrin or cisplatin and incubated (5% CO_2, 37°C) for 24 h. The following day, cells were treated with different concentrations of either reynoutrin or cisplatin and incubated for 24 h. After washing three times with 1XPBS, the cells were stained with 1 mL Hoechst 33342 (10 µg/mL) solution for 15 min in the dark at 37°C. Apoptotic cells (alterations in nuclear morphology) were detected using Hoechst 33342 staining under an inverted fluorescence microscope (Leica, DMIL LED, Germany).

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics software (version 25.0). Numerical data obtained from biochemical analyses and RT-PCR results were analyzed using one-way ANOVA post-hoc Duncan test. Results are presented as the mean ± standard deviation (SD). Additionally, IC_{50} values were calculated using probit analysis. Statistical significance was set at $p < 0.05$.

Results

Reynoutrin reduces MCF-7 and PC-3 cells proliferation

The viability of MCF-7 and PC-3 cells treated with different concentrations of reynoutrin (10-2000 µg/mL) was measured using the MTT assay, and the percentage of viable cells was monitored over 24 h. DMSO vehicle control had no effect on cell viability. These results revealed that reynoutrin reduced the viability of MCF-7 and PC-3 cells in a concentration-dependent manner. Probit analysis revealed that the concentrations required to achieve 50% inhibition (IC_{50}) were 220 µg/mL and 412 µg/mL for MCF-7 and PC-3 cells, respectively. Therefore, in the following experiments, reynoutrin was selected at concentrations corresponding to low, mid-level, and high values relative to its IC_{50}. Concentrations lower than 110 and 206 µg/mL did not cause a significant change in the proliferation of MCF-7 and PC-3 cells, respectively. To evaluate the effects of reynoutrin, MCF-7 cells were treated with concentrations of 110, 220, and 330 µg/mL, whereas PC-3 cells were treated with 206, 412, and 618 µg/mL reynoutrin.

Reynoutrin induced oxidative stress in MCF-7 cells

Figure 1 illustrates the effects of reynoutrin on TAS, TOS, and OSI in MCF-7 cells. In MCF-7 cells, a significant increase in TOS levels and a reduction in TAS levels were observed in the cisplatin-treated group compared with those in the control group ($p < 0.05$). Similarly, reynoutrin induced a concentration-dependent increase in TOS levels and a significant decrease in TAS levels relative to the control group ($p < 0.05$) (Fig. 1a, 1b). Moreover, reynoutrin markedly elevated OSI levels in MCF-7 cells (Fig. 1c). Collectively, these findings suggested that reynoutrin exerted a pro-oxidant effect by enhancing oxidative stress in MCF-7 cells.

Reynoutrin induced oxidative stress in PC-3 cells

Figure 2 illustrates the effects of reynoutrin on TAS, TOS, and OSI in PC-3 cells. In PC-3 cells, a significant increase in TOS levels and a reduction in TAS levels were observed in the cisplatin-treated group compared with those in the control group ($p < 0.05$). Similarly, reynoutrin induced a concentration-dependent increase in TOS levels and a significant decrease in TAS levels relative to the control group ($p < 0.05$) (Fig. 2a, 2b). Notably, a high concentration of reynoutrin (618 µg/mL) exhibited a similar effect on TAS and TOS levels to cisplatin ($p > 0.05$). Moreover, reynoutrin markedly elevated OSI levels in PC-3 cells (Fig. 2c). Collectively, these findings suggest that reynoutrin exerts a pro-oxidant effect by increasing oxidative stress in the PC-3 cells.

Expression results of TNF-α, IL-1β, IL-6, caspase-3, caspase-9, and Bcl-2/Bax mRNA in MCF-7 cells

Analysis of inflammatory markers showed that cisplatin (50 μM) treatment significantly increased the mRNA expression of TNF-α, IL-1β, and IL-6 in MCF-7 cells compared to that in the control group ($p < 0.05$). Conversely, reynoutrin administration (110, 220, and 330 μg/mL) significantly reduced TNF-α, IL-1β, and IL-6 mRNA levels in a concentration-dependent manner ($p < 0.05$; Fig. 3). These RT-PCR results demonstrated the anti-inflammatory potential of reynoutrin via the suppression of pro-inflammatory cytokine gene expression.

Analysis of apoptotic parameters demonstrated that cisplatin significantly elevated caspase-3 and caspase-9 mRNA expression and significantly decreased the Bcl-2/Bax ratio in MCF-7 cells compared with that in the control group ($p < 0.05$). Similarly, medium and high concentrations of reynoutrin (220 and 330 μg/mL, respectively), but not low concentrations (110 μg/mL), induced a significant increase in caspase-3 and caspase-9 expression and a reduction in the Bcl-2/Bax ratio ($p < 0.05$; Fig. 4). Interestingly, the effect of high-dose reynoutrin on caspase-9 expression was similar to that of cisplatin alone. These results indicate that reynoutrin promoted apoptosis in MCF-7 cells in a concentration-dependent manner.

The PCR results showed that reynoutrin exhibited anti-inflammatory activity and induced apoptotic cell death in MCF-7 cells.

Expression levels of TNF-α, IL-1β, IL-6, caspase-3, caspase-9, and Bcl-2/Bax mRNA in PC-3 cells

Analysis of inflammatory markers showed that cisplatin treatment significantly increased the mRNA expression of TNF-α, IL-1β, and IL-6 in PC-3 cells compared with that in the control group ($p < 0.05$). Conversely, administration of reynoutrin (206, 412, and 618 μg/mL) resulted in a significant concentration-dependent reduction in TNF-α, IL-1β, and IL-6 mRNA expression relative to the control group ($p < 0.05$; Fig. 5). These PCR results demonstrate the anti-inflammatory potential of reynoutrin via the suppression of pro-inflammatory cytokine gene expression.

Analysis of apoptotic parameters demonstrated that cisplatin significantly elevated caspase-3 and caspase-9 mRNA expression and significantly decreased the Bcl-2/Bax ratio in PC-3 cells compared with that in the control group ($p < 0.05$). Similarly, reynoutrin induced a significant increase in caspase-3 and caspase-9 expression and a reduction—excluding the low concentration (206 μg/mL)— in the Bcl-2/Bax ratio ($p < 0.05$; Fig. 6). These results indicate that reynoutrin promoted apoptosis in PC-3 cells in a concentration-dependent manner.

RT-PCR results showed that reynoutrin exhibited anti-inflammatory activity and induced apoptosis in PC-3 cells.

Hoechst 33342 staining results

Figure 7 demonstrates the effect of reynoutrin on Hoechst 33342 staining in MCF-7 and PC-3 cells. Reynoutrin induced remarkable morphological alterations in the nuclei of MCF-7 and PC-3 cells compared with those in the control group. Increasing concentrations of reynoutrin led to nuclear shrinkage, fragmentation in both PC-3 and MCF-7 cells, and chromatin condensation, which are the hallmark features of apoptosis. In contrast to the control group, in which the cells displayed intact and healthy blue nuclei, those treated with cisplatin or reynoutrin exhibited enhanced blue fluorescence, which is indicative of the early apoptotic stages. These findings suggest that reynoutrin triggers apoptosis-related morphological changes in MCF-7 and PC-3 cells.

Discussion

Cancer is a leading cause of mortality worldwide. Among various cancer types, breast cancer in women and prostate cancer in men rank as the second most fatal malignancy in their respective populations. Currently, chemotherapy, surgical intervention, and radiotherapy are the primary therapeutic approaches. However, these modalities often exhibit limited efficacy in cases of high incidence or low survival rates, and are frequently associated with severe adverse effects. Therefore, there is an urgent need to explore and develop novel anticancer agents derived from natural sources that are less toxic, more cost-effective, and have fewer side effects [32]. Naturally occurring compounds, particularly flavonoids, are regarded as valuable sources for the development of novel anticancer agents and have demonstrated significant potential in this field [33]. Flavonoids are naturally occurring phytochemicals with diverse pharmacological properties that have been the subject of exponentially increasing research on their pharmacological applications [34]. Flavonoids have demonstrated significant anticancer activities through the induction of oxidative stress, cell cycle arrest, attenuation of inflammatory responses, stimulation of apoptosis and autophagy, and suppression of cancer cell proliferation and invasion [19, 34]. In this study, we investigated the antiproliferative, anti-inflammatory, and apoptotic activities of reynoutrin in the human prostate cancer cell line PC-3 and the human mammary cancer cell line MCF-7.

In the present study, the IC_{50} values of reynoutrin were 110 μg/mL for MCF-7 cells and 206 μg/mL for PC-3 cells. In contrast, Yüksel et al. reported a higher IC_{50} value of 400 μg/mL in MCF-7 cells, suggesting that variations in experimental design, treatment duration, and cellular responsiveness may influence cytotoxic potency [15]. The relatively high IC_{50} values observed in both studies may be attributed to the glycosylated structure of reynoutrin, which is known to limit membrane permeability and reduce intracellular accumulation compared with aglycone flavonoids.

When the balance between prooxidant activity and antioxidant defense is disrupted, ROS levels increase, leading to the accumulation of free radicals. Excessive ROS production results in oxidative stress, which is a key factor in promoting the inflammatory processes associated with cancer development. Flavonoids play a multifaceted role in cancer prevention and treatment, because their antioxidant or pro-oxidant behavior is influenced by their concentration and cellular conditions. Under normal physiological

conditions, flavonoids function as antioxidants, maintaining ROS homeostasis; however, in cancer cells, they can act as potent pro-oxidants, initiating apoptotic pathways and suppressing pro-inflammatory signaling. Although their antioxidant properties protect healthy cells from oxidative stress and potentially lower cancer risk, their pro-oxidant effects at higher concentrations can induce stress in cancer cells, potentially increasing the efficacy of anticancer therapies. This dual action highlights the context-dependent therapeutic potential of flavonoids, which enables them to safeguard normal tissues while rendering cancer cells susceptible to treatment [19, 25]. Research conducted on various cancer cell lines, such as HepG2, HCT116, and OVCAR-3, has shown that different flavonoids can induce oxidative stress in cancer cells by increasing ROS levels. Studies have indicated that daidzein, an isoflavone derived from plants [35], along with flavanone hesperidin [36], both contribute to increased ROS production in MCF-7 cells. Similarly, naringin, a glycoside flavonoid, has been shown to have a comparable effect on PC-3 cells. Elevated ROS levels inhibit cell proliferation and migration, and promote apoptosis in cancer cells. In our study, reynoutrin increased OSI in a dose-dependent manner by increasing TOS and decreasing TAS levels in MCF-7 and PC-3 cells. Notably, the effect of high-dose reynoutrin (616 μg/mL) on oxidative stress in PC-3 cells was similar to that of cisplatin (50 μM). These findings suggest that reynoutrin exhibits a stronger pro-oxidant activity in PC-3 cells than in MCF-7 cells.

When the inflammatory response is not properly regulated, it leads to an increase in the concentration of inflammatory mediators, which can contribute to the development of various chronic diseases including cancer. Inflammation is a hallmark of cancer and plays a critical role in the progression of many types of cancers. Elevated levels of cytokines, such as TNF-α, IL-1β, and IL-6, have been associated with cancer; therefore, cytokine inhibition may represent an important therapeutic target for cancer [20, 22]. Experimental studies have linked TNF-α, IL- 1β, and IL-6 levels with cancer progression. Several studies have demonstrated that various flavonoids, including pelargonidin [37], apigenin [38], quercetin, rutin [39], and luteolin [40], suppress the expression of cytokines, such as TNF-α, IL-1β, IL-6, and inhibit tumor growth. The flavanone hesperetin has been reported to inhibit the secretion of TNF-α, IL-6, and IL-1β in RAW 264.7 cells [41]. Flavon apigenin downregulated TNF-α-associated inflammatory signaling in the A375 human melanoma cell line [38]. Flavonoids, such as gossypin [42, 43], chalcone derivatives [44], and curcumin [45], exhibit anti-inflammatory activity in PC-3 cells by suppressing TNF-α, IL-1β, and IL-6. Our findings support those of previous studies on the expression of proinflammatory cytokines. In the present study, reynoutrin demonstrated antiproliferative activity in both MCF-7 and PC-3 cells by reducing the levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in breast and prostate cancer cells.

Apoptosis, a form of programmed cell death, plays a vital role in the elimination of irreversibly damaged cells from the body. Consequently, it is critical in the context of cancer, and the regulation of pro- and anti-apoptotic proteins has been extensively studied. Among the principal regulators of apoptosis, caspase-3, caspase-9, and Bax act as pro-apoptotic factors, while Bcl-2 functions as an anti-apoptotic factor. Flavonoids, as modulators of the apoptotic pathway, have been shown to promote proapoptotic mechanisms both *in vitro* and *in vivo*. These compounds induce apoptosis in cancer cells by downregulating antiapoptotic proteins and upregulating pro-apoptotic proteins [14]. Numerous studies have demonstrated that genistein in colon cancer cells (HT29) [46], luteolin in breast cancer cells (MDA-

MB-231) [47], naringenin in hepatocellular carcinoma (HepG2) [48], and vitexin [49] in kidney cancer cells upregulate caspase-3., a key pro-apoptotic protein. Similarly, increased Bax levels were reported in cells treated with quercetin, taxifolin, luteolin [47], daidzein [50], vitexin [49], apigenin [51], myricetin [52], naringenin [48], and genistein [53]. In addition, studies have reported that treatment with epigallocatechin [54], myricetin [52], apigenin [51], genistein [53], and daidzein [55] results in significant downregulation of Bcl-2 in prostate cancer, hepatocellular carcinoma, colon cancer, human leukemia, and renal carcinoma cells compared to controls [14]. In our study, RT-PCR analysis revealed significant upregulation of Bax, caspase-3, and caspase-9 gene expression in both MCF-7 and PC-3 cells, which is consistent with the findings of previous studies. Moreover, medium and high concentrations of reynoutrin reduced the Bcl-2/Bax ratio in both cell lines, which is consistent with earlier reports. These results suggest that reynoutrin inhibits anti-apoptotic mechanisms, thereby promoting pro-apoptotic signaling during apoptosis. Apoptosis was assessed using Hoechst staining. Reynoutrin significantly increased nuclear fragmentation in MCF-7 and PC-3 cells in a dose-dependent manner. These findings indicate that reynoutrin induced apoptosis, particularly at higher concentrations.

Conclusion

In summary, our results demonstrated that reynoutrin induces cytotoxic effects by inducing apoptosis and oxidative stress in breast and prostate cancer cells *in vitro* and demonstrated its anti-inflammatory effects. However, further research, especially *in vivo* experiments, are needed to confirm the detailed mechanisms involved. Our report strongly supports the use of reynoutrin as a potential medicinal agent in the development of novel antiproliferative agents.

Declarations

Ethical approval

was not required because it was a cell culture study.

Consent for publication

All authors agreed to the publication of this paper.

Competing interests

The authors declare no competing interests

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Author Contribution

Sedat Gokmen and Irfan Cinar Investigation, Conceptualization, Methodology, Writing- Original Draft, Data Curation, Software, and Validation. All authors have read and approved the final version of the manuscript.

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Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

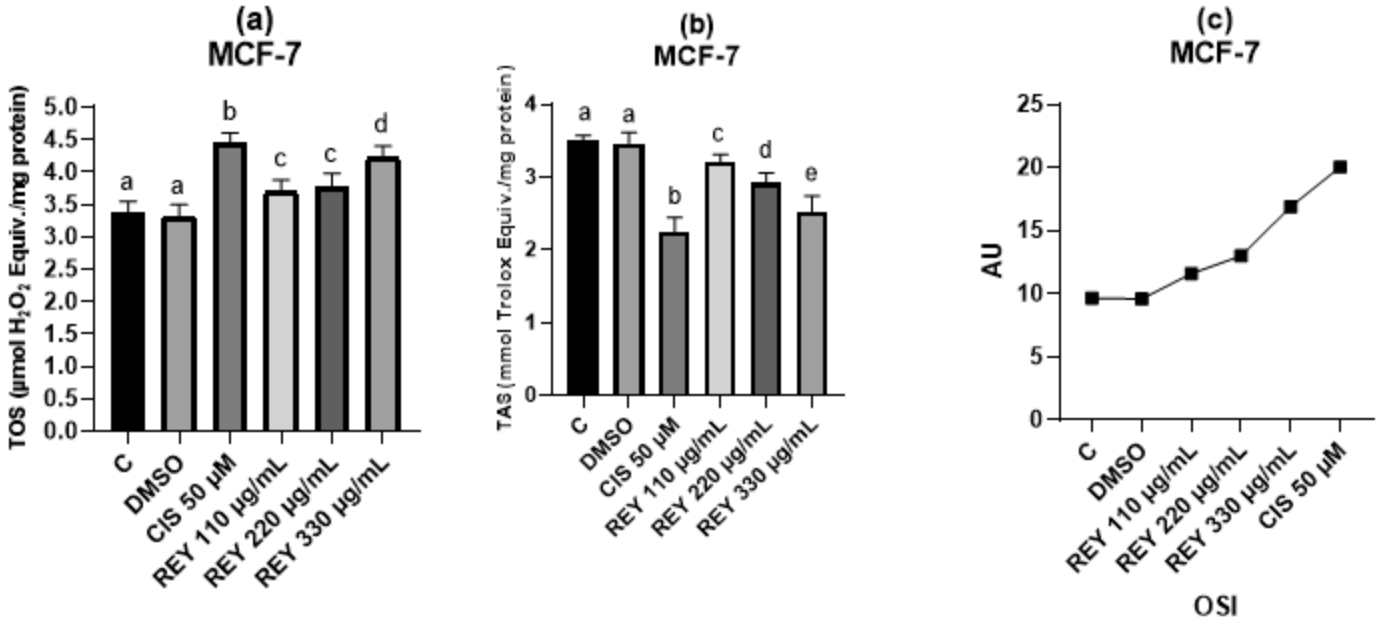


Figure 1

. (a) Total oxidant status (TOS), (b) total antioxidant status (TAS), and (c) oxidative stress index (OSI) levels among different treatment groups following reynoutrin or cisplatin administration in MCF-7 cells. $^{a–d}$ Values not sharing a common superscript differ significantly between groups at $p<0.05$ according to Duncan's Multiple Range Test (DMRT). C: control, Cis: cisplatin, REY: reynoutrin, DMSO: dimethyl sulfoxide.

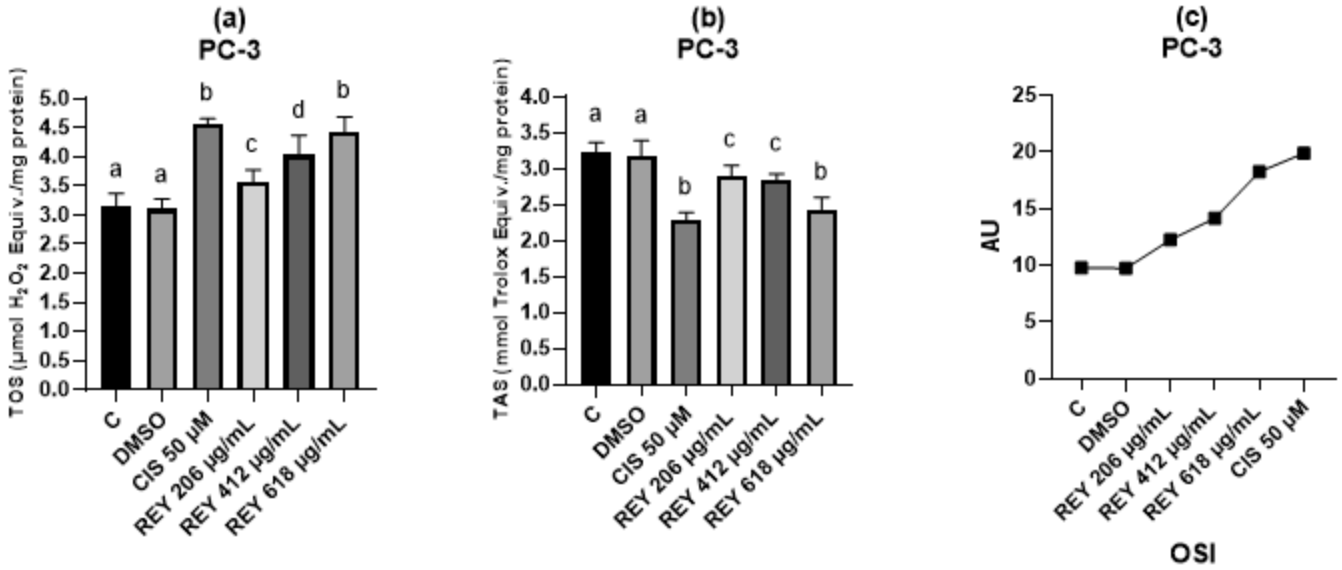


Figure 2

(a) Total oxidant status (TOS), (b) total antioxidant status (TAS), and (c) oxidative stress index (OSI) levels among different treatment groups following reynoutrin or cisplatin administration in PC-3 cells. [a–d] Values not sharing a common superscript differ significantly between groups at p<0.05 according to Duncan's Multiple Range Test (DMRT). C: control, Cis: cisplatin, REY: reynoutrin, DMSO: dimethyl sulfoxide.

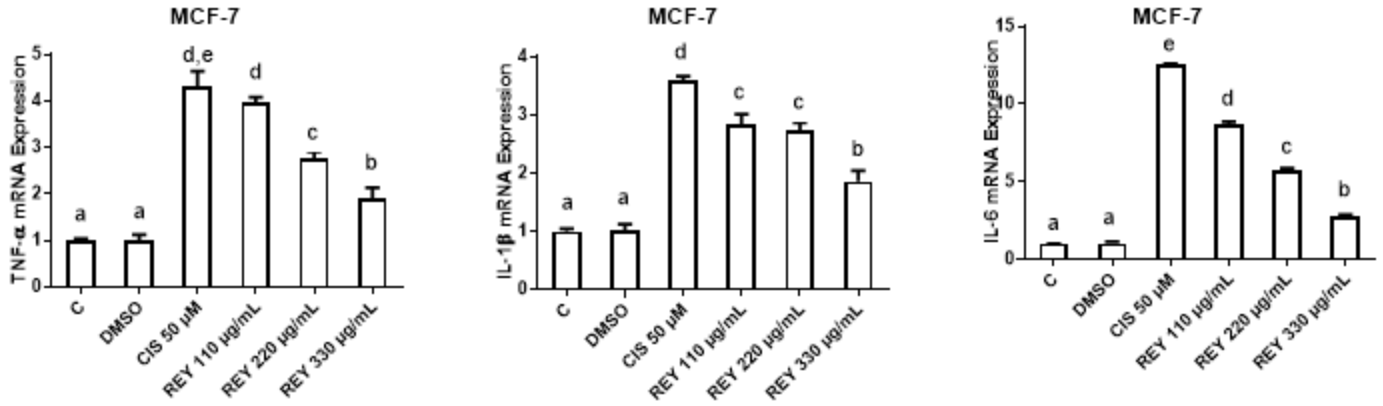


Figure 3

Effect of reynoutrin on inflammation parameters in MCF-7 cells. [a–e] Values not sharing a common superscript differ significantly between groups at p<0.05 according to Duncan's Multiple Range Test (DMRT). C: control, Cis: cisplatin, REY: reynoutrin, DMSO: dimethyl sulfoxide.

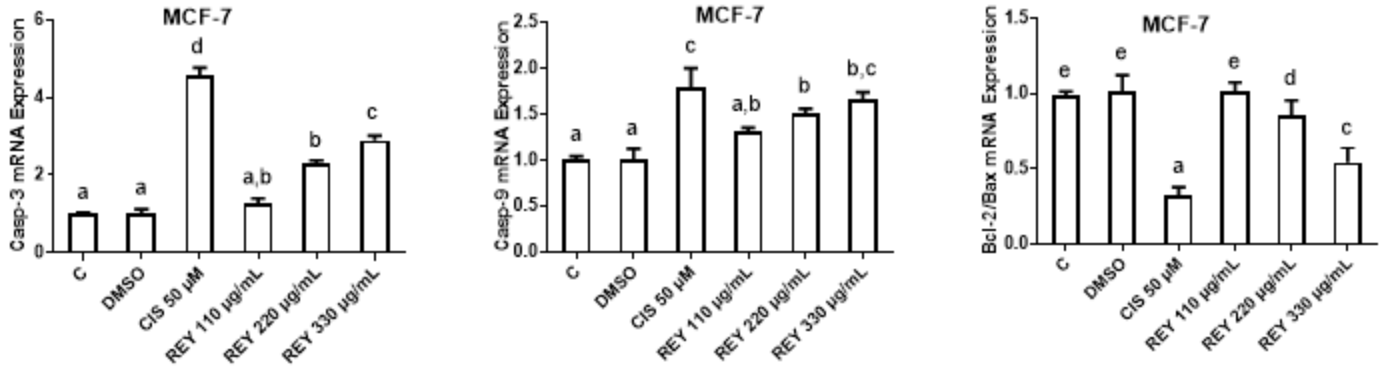


Figure 4

Effect of reynoutrin on apoptosis parameters in MCF-7 cells. [a–e] Values not sharing a common superscript differ significantly between groups at p<0.05 according to Duncan's Multiple Range Test (DMRT). C: control, Cis: cisplatin, REY: reynoutrin, DMSO: dimethyl sulfoxide.

The PCR results showed that reynoutrin exhibited anti-inflammatory activity and induced apoptotic cell death in MCF-7 cells.

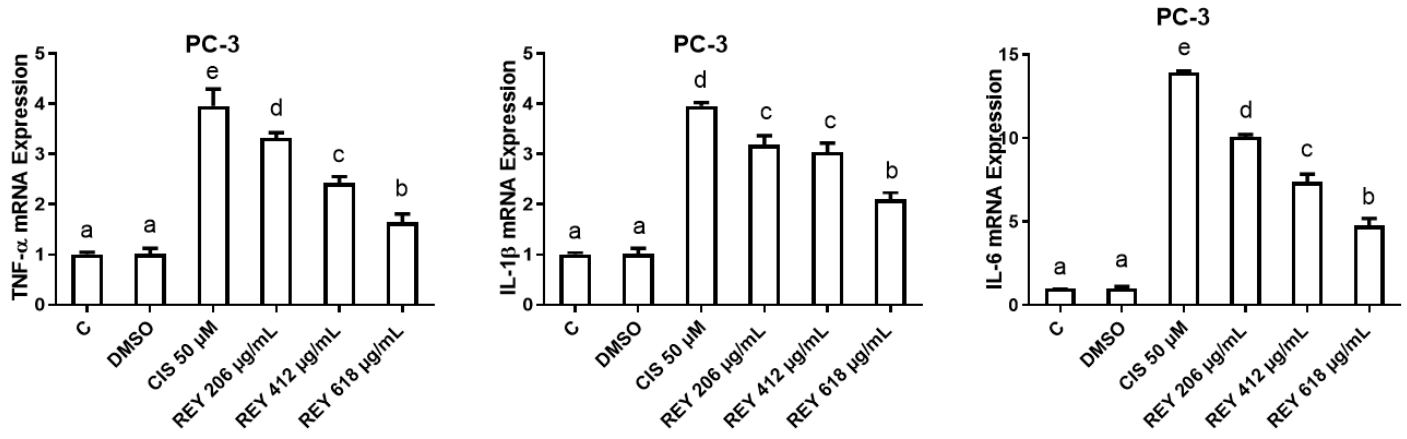


Figure 5

Effect of reynoutrin on inflammation parameters in PC-3 cells. $^{a-e}$ Values not sharing a common superscript differ significantly between groups at $p<0.05$ according to Duncan's Multiple Range Test (DMRT). C: control, Cis: cisplatin, REY: reynoutrin, DMSO: dimethyl sulfoxide.

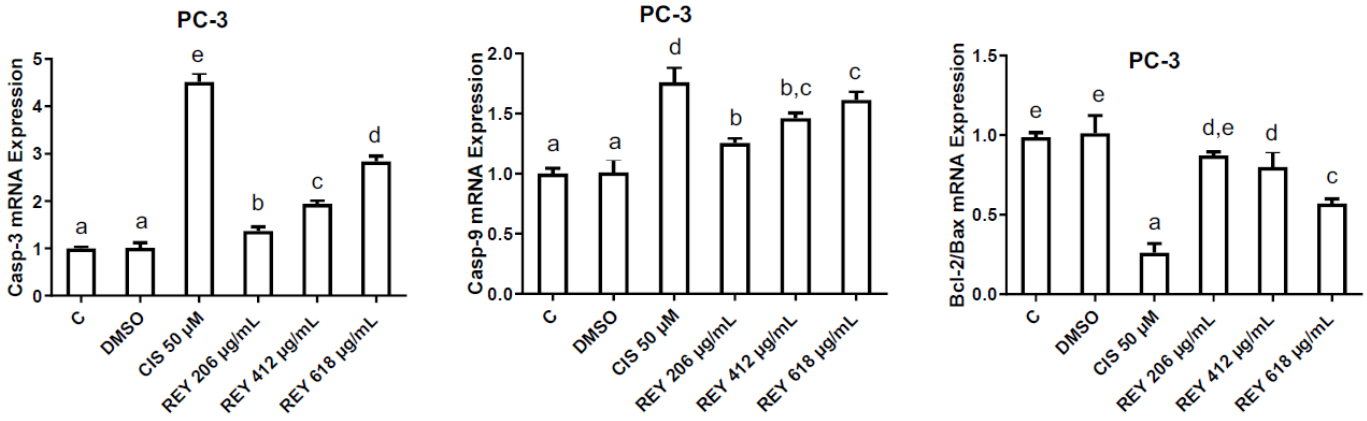


Figure 6

Effect of reynoutrin on apoptosis in PC-3 cells. $^{a-e}$ Values not sharing a common superscript differ significantly between groups at $p<0.05$ according to Duncan's Multiple Range Test (DMRT). C: control, Cis: cisplatin, REY: reynoutrin, DMSO: dimethyl sulfoxide.

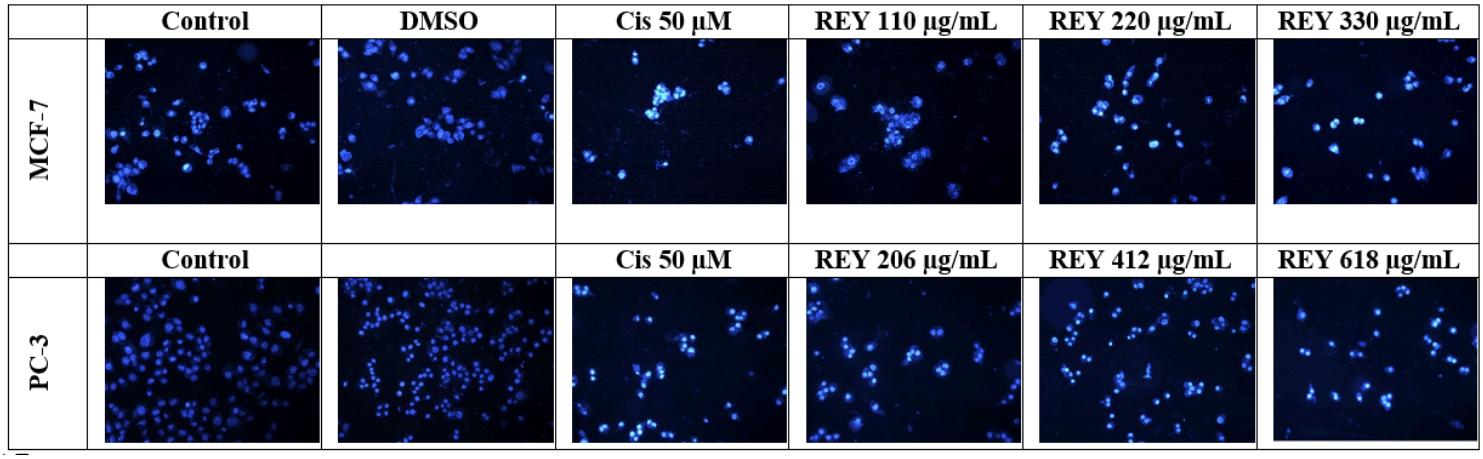


Figure 7

Fluorescent microscope findings after Hoechst 33342 staining in MCF-7 and PC-3 cells treated with reynoutrin (40× magnification). Nuclear morphological changes in reynoutrin-treated MCF-7 and PC-3 cells. MCF-7 breast cancer and PC-3 prostate cancer cells were treated with different concentrations of the compound for 24 h, stained with HO33342, and visualized under a fluorescence microscope. Untreated (control) cells showed healthy blue nuclei, whereas treated cells exhibited apoptotic features including nuclear chromatin condensation, shrinkage, and fragmentation. Early apoptotic events were identified by the bright blue fluorescence of the condensed chromatin. Cis: cisplatin, REY: reynoutrin, DMSO: dimethyl sulfoxide.