

Exploring the Anticancer and Anti-inflammatory Activities of Ferruginol in MCF-7 Breast Cancer Cells

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Abstract: Breast cancer is one of the most prevalent cancers in women, and it has the highest mortality and morbidity worldwide. Breast cancer can be treated by hormone therapies, radiotherapy, surgery, and chemotherapy, but it is often associated with multiple deleterious effects. In this present work, we explored the anti-inflammatory and anticancer effects of ferruginol in MCF-7 cells. The effects of ferruginol on the cell growth and viability of MCF-7 cells were determined by the MTT assay and apoptotic markers. In addition, mitochondrial membrane potential (MMP) status as well as the levels of intracellular reactive oxygen species (ROS), superoxide dismutase, catalase, glutathione, thiobarbituric acid reactive substances (TBARS), caspase-3 and caspase-9 in the ferruginol-treated MCF-7 cells were examined. In addition, expression of inflammation-related proteins such as tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), nuclear factor kappa B (NF- κ B) p65, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) was determined using Western blotting. Our findings showed that ferruginol activated the apoptosis in MCF-7 through a reduction of cell viability. Furthermore, ferruginol-treated MCF-7 cells also showed a decrease of MMP, an increase of ROS, TBARS, caspase-3 and caspase-9, as well as a reduction of antioxidant proteins. Ferruginol treatment is also downregulated the expression of inflammatory modulators such as TNF- α , iNOS, COX-2, NF- κ B p65, and IL-6 in MCF-7 cells. In conclusion, ferruginol inhibits the inflammation and activated apoptosis by modulating the expression of inflammatory and apoptotic markers. Therefore, ferruginol may serve as a potential curative agent for breast cancer.

Keywords: Ferruginol, Inflammation, Breast cancer, Caspase, Apoptosis

1 Introduction

Breast cancer is an important cause of fatality in women globally^[1]. Currently, the treatments of breast cancer include hormone therapies, chemotherapy, surgery, and radiotherapy^[2]. Although patients primarily responded to these treatments, treatment resistance frequently happened and no solutions are available for patients with advanced breast cancer. Thus, it is necessary to look for new and better chemotherapeutic compounds for the management and prevention of breast cancer^[3]. Since the drugs which are presently in use would lead to unwanted side effects, harmless bioactive compounds should be considered and developed as curative agents^[4].

Inflammation is a protection mechanism in normal host that is generally activated to attack foreign substances and in response to different systemic damages^[5,6]. The activation of inflammatory cells causes the overproduction of different types of inflammatory proteins, principally nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) which intensify inflammatory processes to either eradicate invading foreign substances or modify injured tissue structures. Nevertheless, augmented release of the inflammatory proteins for a prolonged period of time may engender harmful effects to various organs^[7].

Chronic inflammation is associated with the multiple stages of tumorigenesis, including the transformation of cells, progression, multiplication, metastasis, angiogenesis, and invasion. The stimulators of inflammation can elevate cancer risks. For instance, *Helicobacter pylori* induces gastric sarcoma and mucosal lymphoma, papillomavirus induces cervical sarcoma, and hepatitis virus increases the risk for the liver carcinoma. Furthermore, autoimmune diseases like inflammatory bowel disease may trigger the development of colon cancer, and some uncertain conditions of inflammation may promote the risk of prostate cancer. Inflammation often results in tissue injury, which leads to cell multiplication and tissue repair. The cell multiplication in this circumstance is normally related to metaplasia, a reversible but abnormal alteration to the cells. The dysplasia is a disorder of cell multiplication that often leads to uncharacteristic cell multiplication, and dysplasia is considered the earliest phase of sarcoma because it normally occurs in the surrounding of neoplasm site^[8,9].

Apoptosis is an important physiological process that regulates tissue homeostasis, and it plays an important role in the pathogenesis of different illnesses^[10]. Dysregulation of apoptosis is the main contributor to the pathomechanism of carcinogenesis. Apoptosis is regulated by caspases, which are stimulated through two pathways, namely, the intrinsic pathway that depends on the release of mitochondrial

factors and extrinsic pathway that involves stimulation of cell-surface death receptors^[11]. In several pathological circumstances, the disease condition occurred due to the irregular and malfunctioning of apoptotic mechanisms^[12]. During carcinogenesis, malfunctioned apoptosis is the most apparent cause that tumor cells cannot die due to unregulated proliferation^[13]. Thus, targeting dysregulated apoptosis and the uncontrolled growth of malignant cells have become the key objective in the anticancer approach^[14].

The development and improvement of new plant-derived compounds are some of the important goals in anticancer research. Throughout the history of research and observance of conventional remedies for their anticarcinogenic effects in modern decades, the drugs that have clear mechanism of action might lend a helping hand in the improvement of innovative anticancer treatments^[15]. Ferruginol, which belongs to the class of diterpenoids, has been proven to possess a wide array of pharmacological effects such as antifungal, mitocidal, antiulcerogenic, antibacterial, antiplasmodial, and cardioactive and antioxidative effects^[16-21]. Moreover, a few of previous investigations also proved that the ferruginol possesses antitumor activities against prostate and non-small-cell lung cancers^[16,22]. Presently, to the best of our understanding, no findings pertaining to the role of ferruginol in mediating inflammation and apoptosis of MCF-7 cells have been presented. In view of this, we explored the effects of ferruginol on suppressing inflammation and apoptosis of breast cancer cells as well as the related mechanisms in the current study.

2 Materials and methods

2.1 Chemicals

Ferruginol was obtained from Sigma-Aldrich Chemicals Pvt. Ltd. Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), MTT assay kit, and Dulbecco's Modified Eagle Medium (DMEM) were purchased from HiMedia Lab Ltd. All chemicals used in this study were of the analytical grade. The primary

antibodies for TNF- α , IL-6, nuclear factor kappa B (NF- κ B) p65, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and beta (β)-actin were acquired from Santa Cruz Biotechnology. Caspase3 and -9 assay kits were acquired from Bio-Rad, Hercules, California, USA.

2.2 Cell culture

The MCF-7 cells were grown in DMEM, supplemented with antibiotics and 10% FBS. The cells were maintained in an incubator with a temperature of 37°C and 5% carbon dioxide. Approximately 2.5×10^4 – 1.0×10^5 MCF-7 cells were seeded in each well for each experiment.

2.3 MTT assay

The cytotoxic effect of ferruginol on MCF-7 cells was determined using MTT assay. MCF-7 cells were trypsinized and 1×10^5 cells were plated in each well of a 96-well plate. Cells were cultured and then treated with a range of doses of ferruginol (6, 12, 24, and 48 μ M). The cells which were treated with 0.01% DMSO instead of ferruginol served as the control. In this experiment, the cells were cultured in serum-free medium and incubated for 24 h at 37°C. After that, 20 μ L of MTT was mixed in PBS at a concentration of 5 mg/ml and the solution was added to each well. The cell plates were kept for 4 h at 37°C. Formazan crystals that were formed later were dissolved in 100 μ L of DMSO and optical density was measured at 490 nm on a microplate reader. The percentage of MCF-7 cell viability was calculated^[23].

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

The 50% inhibitory concentration (IC_{50}) of ferruginol that suppresses the cell growth of MCF-7 cells by 50% was determined using GraphPad Prism 7.0 (GraphPad Software, CA, USA).

2.4 Measurement of intracellular ROS using dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescent staining technique

The level of ROS was measured according to a method described earlier^[24]. Briefly, $1.0 \times$

10^5 cells were seeded into each well of a 6-well plate. The cells were cultured overnight, and subsequently, the cells were treated with 6 and 12 μ M of ferruginol for 12 h. Instead of ferruginol, control cells received 5 μ M of DMSO. After the incubation, the cells were incubated with 20 μ M of DCFH-DA for 30 min. Following the removal of unwanted DCFH-DA, the cells were washed with PBS and observed under a fluorescence microscope.

2.5 Estimation of mitochondrial membrane potential (MMP)

MMP was estimated using fluorescent dye rhodamine (Rh)-123^[25]. Briefly, 1×10^5 cells were cultured in each well of a 6-well plate and allowed to adhere overnight. Next, MCF-7 cells were incubated with 6 and 12 μ M of ferruginol, whereas control cells were treated with 5 μ M of DMSO at 37°C for 24 h. Following the treatment, the cells were trypsinized and rinsed with PBS, and then, 5 μ g/ml of Rh-123 was added. MMP was measured and estimated at 485/530 nm in a spectrofluorometer.

2.6 Assays of lipid peroxidation and antioxidant proteins

In this experiment, 1×10^5 MCF-7 cells were seeded in each well of a 6-well plate. The cells were treated with 6 μ M or 12 μ M of ferruginol, while control cells were treated with 5 μ M of DMSO. Following treatment, the cells were subject to a 24-h incubation. Afterward, cells were washed, lysed, and then centrifuged at 12,000 rpm and 4°C for 15 min. The supernatant was obtained and used for the measurements of oxidative stress markers. The level of thiobarbituric acid reactive substances (TBARS) was measured using the methodology described by Ohkawa *et al.*^[26]. Similarly, the measurements of other oxidative stress parameters such as superoxide dismutase (SOD) activity^[27], catalase (CAT) activity^[28], and glutathione (GSH) concentration^[29] were performed in accordance with previously described techniques.

2.7 Caspase assay

In this experiment, 1×10^5 MCF-7 cells were seeded in each well of a 6-well plate. In brief, after treatment with either 6 and 12 μM of ferruginol or 5 μM of DMSO, the collected cells were lysed and subjected to caspase-3 and caspase-9 measurement. The levels of caspase-3 and caspase-9 were measured using colorimetric assay kits. Following incubation at 37°C for 4 h, the development of *p*-nitroanilide was observed and the corresponding absorbance was measured spectrophotometrically at 405 nm.

2.8 Western blotting

In this experiment, 1×10^5 MCF-7 cells were cultured in each well of a 6-well plate. The cells were rinsed with chilled PBS and then lysed in lysis buffer which was pre-chilled at 4°C . After centrifugation, the supernatant containing proteins was collected and stored. Protein concentration was determined using Lowry *et al.* method^[30]. Before Western blotting, the proteins were denatured at 95°C . Approximately 40 μg of protein of each sample was used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gel were then transferred to the polyvinylidene fluoride (PVDF) membrane. After protein transfer, the PVDF membrane was rinsed with Tris-buffered saline and incubated with primary antibodies at 4°C . The membrane was incubated with appropriate secondary antibodies. Protein bands were visualized using enhanced chemiluminescence reagents. Densitometric analysis was performed. Values were corrected with β -actin as a control.

2.9 Statistical analysis

In the present study, the data of triplicate values were expressed as mean \pm standard deviation. The statistical variations among the test groups were analyzed using one-way analysis of variance coupled with a *post hoc* test called Duncan's multiple range test. The statistical analyses in this study were performed using Statistical Package for Social Sciences, version 19 (SPSS Inc., Chicago, IL, USA). The differences with $P < 0.05$ are regarded as statistically significant.

3 Results

3.1 Effect of ferruginol on the viability of MCF-7 cells

The effect of ferruginol on the cell viability of MCF-7 cells was examined, and the results are shown in **Figure 1**. The ferruginol-treated MCF-7 cells showed a marked reduction in cell viability after being treated with increasing ferruginol concentrations (6, 12, 24, and 48 μM) for 24 h and the percent of viable cells were noticeably decreased. The treatment with ferruginol has reduced the cell viability of MCF-7 cells in a dose-dependent manner, indicating that ferruginol triggered apoptosis in MCF-7 cells. At treatment for 24 h, the IC_{50} of ferruginol was found to be 12 μM in MCF-7 cells. Thus, both 6 μM and 12 μM of ferruginol were opted to be used in subsequent experiments.

3.2 Effect of ferruginol on ROS production

The effect of ferruginol on the intracellular ROS production in MCF-7 cells was investigated using the DCFH-DA fluorescent staining technique. As shown in **Figure 2**, the treatment with the ferruginol (6 and 12 μM) augmented the intensity of green fluorescence in MCF-7 cells, which was higher than that in the control. This indicates that ferruginol could activate the intracellular ROS

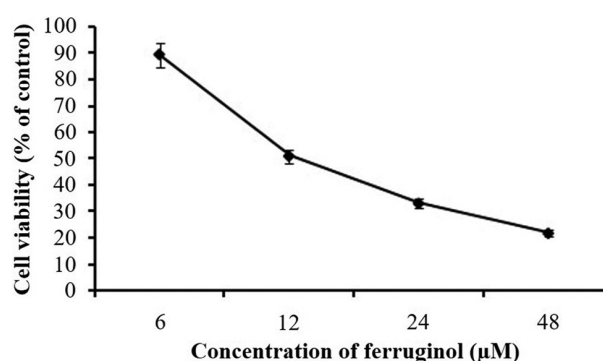


Figure 1. Cytotoxic effect of ferruginol on MCF-7 cells. The viability of MCF-7 cells was determined by MTT assay. Ferruginol inhibited the growth of MCF-7 cells in a dose-dependent manner. The experiments were performed in triplicate and the results were expressed as mean \pm standard deviation.

generation in MCF-7 cells. However, the cells that were not treated with ferruginol showed very dim fluorescence. This result is consistent with the apoptosis-triggering potential of ferruginol in MCF-7 cells.

3.3 Effect of ferruginol on MMP

The effect of ferruginol on the MMP of MCF-7 cells was examined using the Rh-123 fluorescent staining. **Figure 3** displays the photographs which were stained with Rh-123. No changes in the MMP were identified in the untreated cells of the control group. The significantly reduced fluorescence intensity in the ferruginol-treated MCF-7 cells showed that the MMP was diminished after ferruginol treatment. MMP was drastically decreased in the cells treated with 6 and 12 μ M of ferruginol when compared to the control. Fluorescent

microscopic imaging of ferruginol-treated MCF-7 cells, as shown in **Figure 3**, showed that the ferruginol treatment may potentially cause the loss of MMP. Thus, we speculate that MMP diminution triggered by the ferruginol in MCF-7 cells may lead to cell death.

3.4 Effect of ferruginol on the levels of lipid peroxidation and antioxidants

The treatment with ferruginol has markedly altered the levels of TBARS as well as antioxidant proteins in MCF-7 cells as compared to the untreated cells in the control group. **Figure 4** demonstrates that the levels of TBARS and antioxidant proteins such as SOD, CAT, and GSH in ferruginol-treated MCF-7 cells and untreated cells. Untreated MCF-7 cells in the control group manifested a reduction in the TBARS level and an increase in the levels of antioxidant

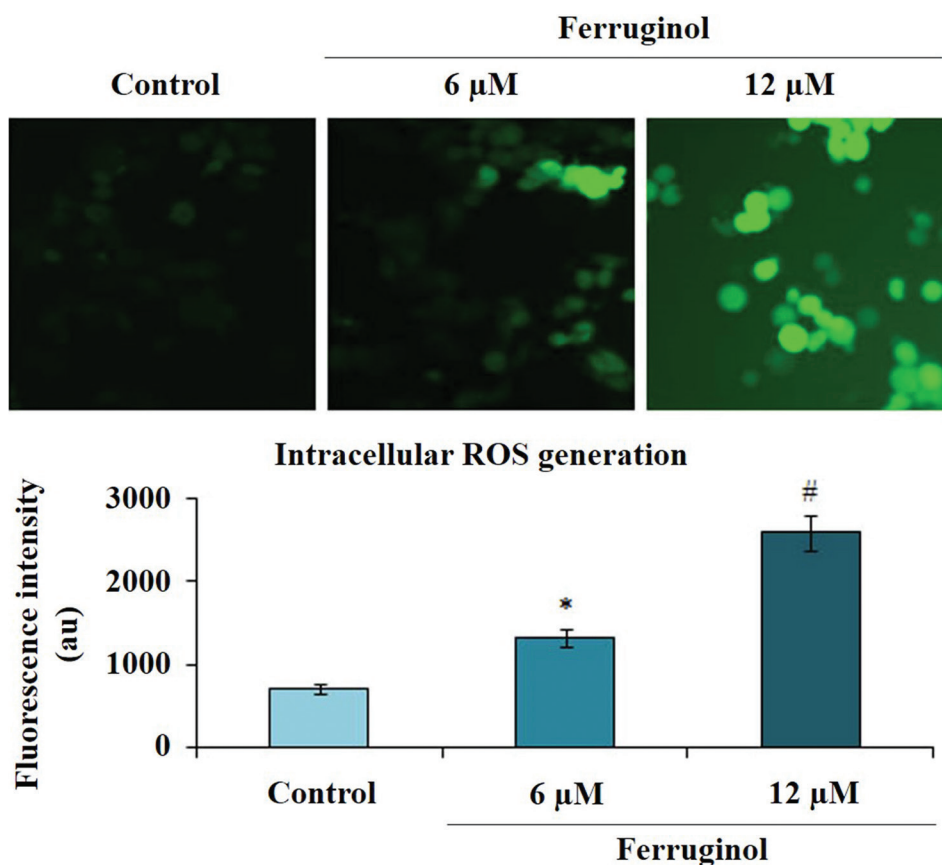


Figure 2. Effect of ferruginol on the intracellular reactive oxygen species (ROS) generation in MCF-7 cells. The intracellular ROS generation was measured using the dichloro-dihydro-fluorescein diacetate fluorescent staining technique. The presented data, which are representative of triplicate values, were expressed as mean \pm standard deviation. * $P < 0.05$ and # $P < 0.01$ compared with the control.

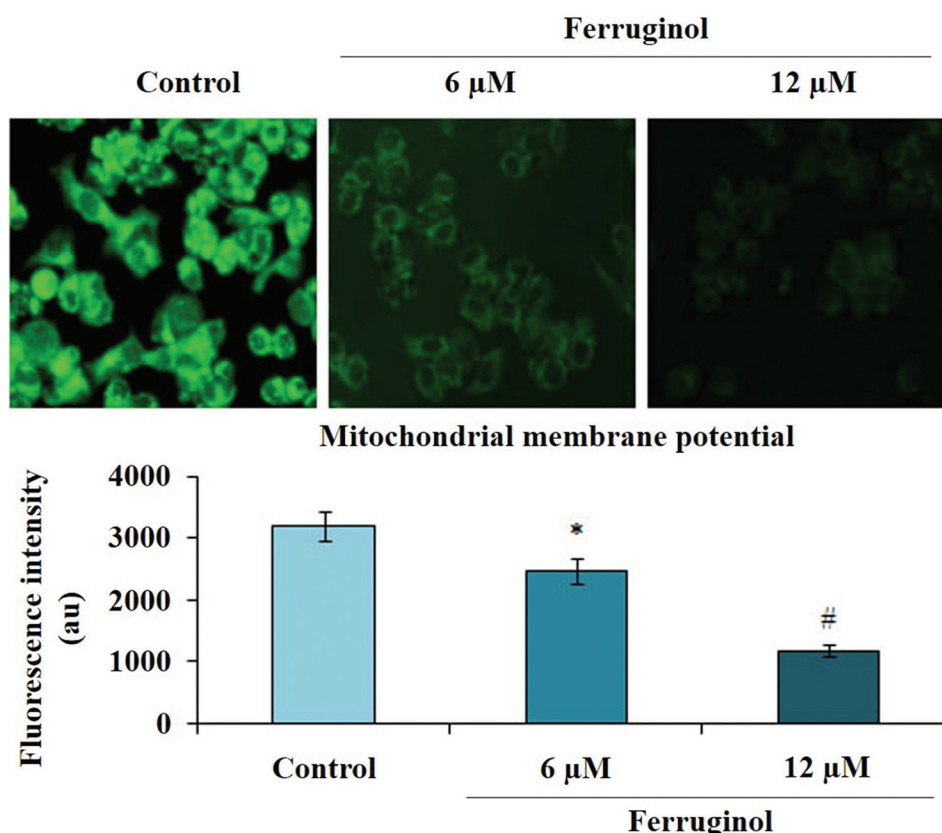


Figure 3. Effect of ferruginol on mitochondrial membrane potential (MMP) in MCF-7 cells. Estimation of MMP was performed using rhodamine (Rh)-123 fluorescent staining. The data, which were representative of triplicate values, were expressed as mean±standard deviation. * $P < 0.05$ and # $P < 0.01$ compared with the control.

proteins. Conversely, we also noticed that ferruginol-treated MCF-7 cells showed higher levels of TBARS and lower levels of antioxidant proteins in comparison to control. The decrease of antioxidant proteins attributed to ferruginol treatment may indicate the apoptosis-triggering potential of ferruginol in breast cancer cells.

3.5 Effect of ferruginol on caspase-3 and caspase-9

The effect of ferruginol in the activities of caspase-3 and caspase-9 in MCF-7 cells was investigated. As shown in **Figure 5**, the alterations in activities of caspase-3 and caspase-9 in the ferruginol-treated MCF-7 cells were noticeable. The untreated cells in the control group showed low activities of caspase-3 and caspase-9, whereas the ferruginol treatments significantly increased the activities of caspase-3 and caspase-9 in a dose-dependent manner.

3.6 Effect of ferruginol on the expression of inflammation-related proteins

Figure 6 shows the expression of inflammation-related proteins such as TNF- α , IL-6, NF- κ B p65, iNOS, and COX-2 in ferruginol-treated MCF-7 cells and control. Ferruginol-treated MCF-7 cells displayed reduced levels of these inflammation-related proteins relative to the untreated cells of control. Consequently, it was proved that the ferruginol treatment was noticeably suppressed the protein expressions of inflammatory modulators.

4 Discussion

Cancer is one of the most devastating illnesses in the world which is characterized by unregulated cell growth, diminished apoptosis, and metastasis^[31]. Breast cancer is one of the most common cancer in women and leads to

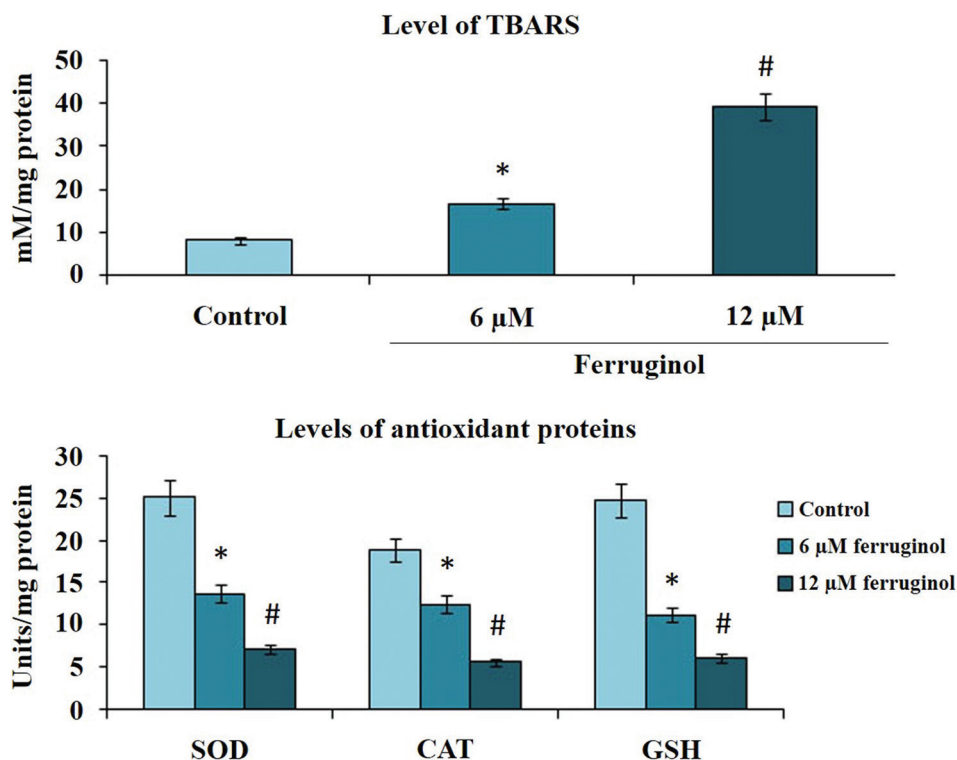


Figure 4. Effects of ferruginol on the levels of lipid peroxidation and antioxidant proteins in MCF-7 cells. Thiobarbituric acid reactive substance (TBARS) is a marker of lipid peroxidation, whereas superoxide dismutase, catalase, and glutathione are antioxidant proteins. The data, which were representative of triplicate values, were expressed as mean \pm standard deviation. * $P < 0.05$ and # $P < 0.01$ compared with the control.

the highest rates of mortality and morbidity in women worldwide^[32].

Developing and improving therapies targeting breast cancer cells by means of compounds that do not interfere with or affect normal cells is an arduous challenge in the field of cancer drug development^[33]. Due to the limited achievement of standard clinical treatments such as surgery, chemotherapy, and radiotherapy, which are also marked by the elevated mortality and morbidity rates as well as serious side effects such as leukopenia, carcinogenicity, bone marrow depression, and anemia, it is imperative to develop novel cancer treatments^[34]. Natural compounds derived from herbs have been shown to be outstanding and reliable sources for the synthesis of novel anticancer compounds because of minimal side effects as compared to synthetic drugs^[35]. Other studies also supported that natural products originated from plants with medicinal values demonstrate

antimetastatic, anti-invasive, antiangiogenic, and antiproliferative activities^[36,37].

MTT assay is an *in vitro* test that is widely accepted for evaluating the cytotoxic properties of drugs. In the present study, the cytotoxic effect of ferruginol at various concentrations against MCF-7 cells was investigated. We showed that ferruginol was capable to suppress the growth of MCF-7 cells and had potent cytotoxicity on the breast cancer cells in a dose-dependent manner, indicating anticancer activity possessed by ferruginol. Previous studies also reported that ferruginol inhibited the growth of MDA-T32 cells, a papillary thyroid carcinoma cell line^[38].

Mitochondria play a crucial part in triggering apoptosis in mammalian cells. Alterations to certain essential markers that are implicated in mitochondrial destruction such as ROS, MMP, TBARS, SOD, CAT, and GSH may potentially elicit chain reactions in mitochondrial apoptotic

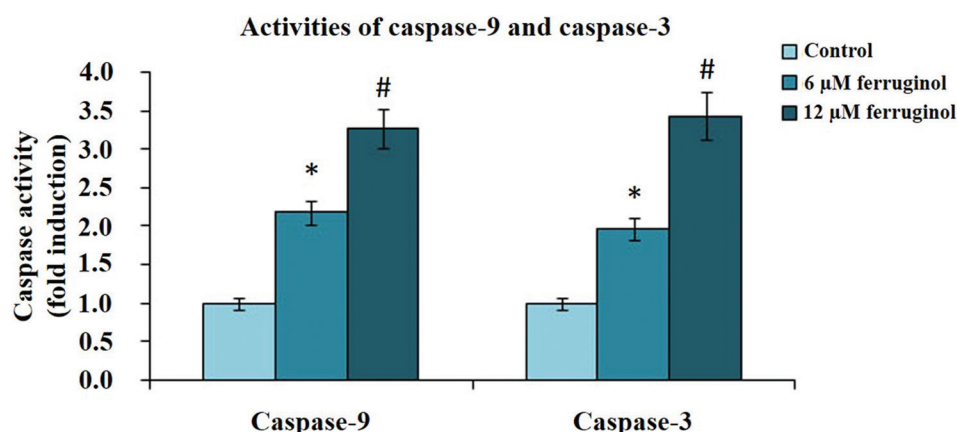


Figure 5. Effect of ferruginol on caspase activities in MCF-7 cells. The activities of caspase-3 and caspase-9 were measured by the colorimetric method. MCF-7 cells were treated with ferruginol (6 and 12 μ M) for 24 h and the levels of caspase-3 and caspase-9 were analyzed. The data, which were representative of triplicate values, were expressed as mean \pm standard deviation. * $P < 0.05$ and # $P < 0.01$ compared with the control.

signaling pathways^[39,40]. Both MMP and ROS are the two main markers of mitochondrial damage. The current study demonstrated an elevation of ROS level and reduction of MMP after ferruginol treatment, in comparison to the control. The overproduction of intracellular ROS may lead to the oxidation of membrane-bound lipids, thereby, altering the antioxidant enzyme status that eventually results in the diminution of MMP^[41]. As a result, loss of MMP and rupture of the mitochondrial membrane could lead to the opening of mitochondrial permeability transition pore and activation of caspase, which eventually activate the mitochondrial apoptotic signaling pathway^[42]. In this regard, a recent study showed that ferruginol aggravates mitochondrial dysfunction by rising intracellular ROS generation, reducing MMP, and ultimately activating caspases in human thyroid cancer cell lines^[38].

Cell apoptosis can be activated by different types of drugs and also chemical and physical mediators. The stimulation of apoptosis has been regularly described as a useful mechanism and approach in anticancer treatment^[43]. Cell apoptosis is initiated when the cell is stimulated by various external apoptotic signals which inactivate several main proteases in the cytoplasm, cytoskeleton, and nucleus^[44]. Caspase-3 is present in an inactive form in the cytoplasm. Caspase-9 is implicated

in the activation of the mitochondrial apoptotic signaling pathway and its activity has been identified in numerous types of cancer cells^[45]. In the present study, our findings demonstrated that the activities of caspase-3 and caspase-9 were elevated following ferruginol treatment in breast cancer cells. Ho *et al.* reported that ferruginol could activate apoptosis in lung cancer cells^[22]. Likewise, Xiong *et al.* also showed that apoptosis engendered cell death in ovarian cancer cells^[46]. Thus, our results agree with earlier literature^[46,47]. In addition, our findings may support that ferruginol can elicit caspase-dependent apoptosis in MCF-7 breast cancer cells.

Research discovery in the past two decades pointed out that at the molecular level, many chronic diseases and cancers are caused by dysregulated inflammatory reactions^[48]. Of note, NF- κ B which is a pro-inflammatory transcription factor is one of the most important linkers between carcinogenesis and inflammation. NF- κ B is a ubiquitous and evolutionarily preserved transcriptional factor that controls the expression of genes implicated in proliferation, survival, transformation, angiogenesis, invasion, and metastasis of cancer cells^[36]. Apart from that, NF- κ B exists in different isoforms either as homo- or heterodimers^[7]. The most important isoform of NF- κ B is the heterodimer of p50 and

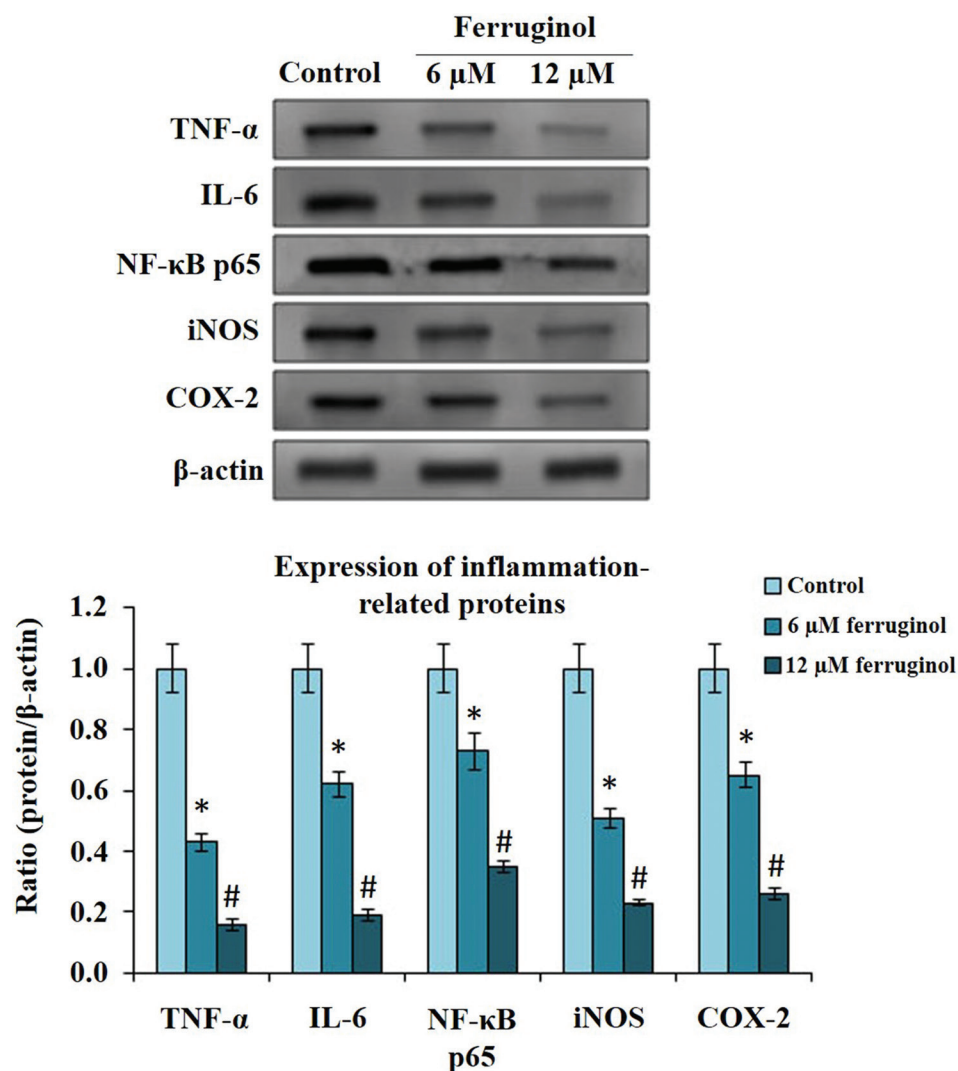


Figure 6. Anti-inflammatory effect of ferruginol in MCF-7 cells. Lines were examined by Western blotting technique. The protein expression of tumor necrosis factor- α , interleukin-6, nuclear factor- κ B p65, inducible nitric oxide synthase, and cyclooxygenase-2 in ferruginol-treated MCF-7 cells was analyzed using Western blotting. β -actin was used as a loading control, and the values of densitometric analysis were corrected with β -actin. The data, which were representative of triplicate values, were expressed as mean \pm standard deviation. * $P < 0.05$ and # $P < 0.01$ compared with the control.

p65. NF- κ B is usually situated in the cytoplasm with its inhibitory protein, I κ B α . On activation, NF- κ B is released from I κ B α protein due to ubiquitination, phosphorylation, and consequent degradation of I κ B α by the proteasome^[49]. Subsequently, I κ B α liberated NF- κ B which is then translocated to the nucleus, stimulating expression of different pro-inflammatory proteins such as COX-2, iNOS, TNF- α , and IL-6^[50-52]. In the current study, ferruginol significantly decreased

the expression levels of these inflammatory mediators, indicating anti-inflammatory effect of ferruginol. Thus, these results are consistent with a recent study which demonstrated the inflammation-suppressing effect of ferruginol on SK-Mel-28 human malignant melanoma cells^[53].

5 Conclusion

Taken together, ferruginol inhibits cell proliferation, augments intracellular ROS

generation, reduces MMP, and decreases levels of antioxidant proteins in MCF-7 cells. Furthermore, ferruginol increases the expression of apoptotic proteins such as caspase-3 and caspase-9. Moreover, ferruginol also possesses anti-inflammatory activity as evidenced by a reduction of inflammation-related protein expression. In view of the beneficial impacts on MCF-7 cells, as shown in the present study, ferruginol is a potential curative agent for breast cancer. More investigations are warranted to further corroborate our findings.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

T.R. conceived and designed the experiments and contributed reagents/materials/analysis tools. S.K. performed the experiments. V.P. and S.D. analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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