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The Effects of Genistein on Peroxiredoxin-4 and Oxidative Stress in Lung Cancer Cell Line A549

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ABSTRACT

Background and Objective: Genistein is a substance from the group of isoflavones that has been reported to have anti-cancer properties in various cancers. This study was designed to investigate the effect of genistein on oxidative stress markers in lung cancer cell line A549.

Methods: In this experimental study, 0, 20, 40, 60, 80, and 100 µM genistein concentrations were used to treat A549 cells and then cell viability was measured by MTT assay after 24 and 48 hours. After selecting the IC50 dose and performing the treatment, TAC, TOS and CAT tests were measured manually and PRx4, SOD and GPx tests were measured by the kit.

Findings: The IC50 dose of genistein for A549 cells was 40 µM in 24 h. The 40 µM dose of genistein was significantly different for TAC compared with the control (0.05±0.01, 0.07±0.01 mmol/mg, respectively). However, it caused a significant increase in TOS (0.03±0.004, 0.01±0.002 mmol/mg, respectively) and OSI (0.57±0.08, 0.18±0.06, respectively) compared with the control group ($p<0.05$). Our results also showed that SOD (0.95±0.02, 2.56±0.19 UI/mg, respectively), CAT (0.45±0.11, 1.23±0.18 UI/mg, respectively) and PRXD4 (18.35±1.45, 42.83±3.75 pg/mg respectively) were significantly reduced in the treated groups compared to the control group ($p<0.05$). GPx was significantly reduced at a dose of 60 µM (85.28±4.36, 141.59±12.36 UI/mg, respectively) compared to the control group ($p<0.05$).

Conclusion: The results of the study showed that genistein reduces viability rate and induces oxidative stress in A549 cancer cells.

Keywords: *Genistein, Oxidative Stress, A549, Lung Neoplasms.*

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Introduction

According to the World Health Organization report in 2018, lung cancer is one of the most common types of cancer worldwide and has the highest mortality rate (1.76 million deaths) compared to other malignant tumors. Lung adenocarcinoma is more common in people over 50 years of age, and smoking is the most important risk factor for this disease (1). Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are two main groups of lung cancer, and NSCLC group includes 85% of cases (2, 3). Oxidative stress is one of the main characteristics of tissue and cell damage. Many studies have shown the importance of antioxidant and pro-oxidant markers in various cancers (4). Furthermore, the beneficial role of an antioxidant-rich diet in preventing cancer has been proven. Oxidative stress occurs when there is an imbalance between the antioxidant/oxidant system and the cell cannot neutralize pro-oxidants and free radicals. This means that with the increase in oxidizing factors, the antioxidant defense systems are not enough to balance them (1).

Reactive oxygen species (ROS) are the main class of radicals produced in cells and include hydroxyl, perhydroxyl, peroxyl and superoxide radicals and non-free radical species such as singlet oxygen and hydrogen peroxide (5). Recent studies have shown conflicting results about the role of ROS in cancers. On the one hand, the increase in ROS can suppress cancer and apoptosis by activating caspases and deactivating various signaling pathways in some cancers (6-10). On the other hand, several research findings have shown that increased ROS levels may lead to mutagenesis through DNA damage, especially if the accumulation of DNA damage is accompanied by a defect in the repair system or an apoptotic pathway (11). Pro-oxidants are continuously produced in living cells; thus, several antioxidant defense systems are required to deal with them and prevent oxidative stress. These defense systems contain antioxidants such as vitamin E, vitamin C and GSH and antioxidant enzymes such as CAT, GPx and SOD (12, 13). SOD enzyme moderates cellular oxidative stress by removing superoxide radicals and converting them into hydrogen peroxide. Then, Gpx and CAT remove hydrogen peroxide through GSH oxidation and water production, respectively (14).

Genistein belongs to the group of isoflavonoids with the formula $C_{21}H_{20}O_{10}$ and is found in plant-based foods such as soy (15). Numerous clinical tests and reviews have predicted the therapeutic role of genistein in different cancers. The anticancer properties of genistein have been investigated on breast, colorectal and liver cancers under in vitro conditions (16-18). There are conflicting results regarding the effect of genistein on oxidative stress in various cancers (19-21). This led us to design a new study to gain insight into the effect of thymoquinone on oxidative stress markers on lung cancer cells to determine the antitumor mechanism of thymoquinone. Therefore, the present study was conducted to investigate the effects of genistein on oxidative stress by measuring the activities of TOS, TAC, PRX4, CAT, GPx and SOD enzymes.

Methods

This interventional study was conducted after getting the approval of the ethics committee of Hamadan University of Medical Sciences with code IR.UMSHA.REC.1399.257.

Cell culture: The A549 cell line (Human Non-Small Lung Cancer Cell Line) was purchased from Pasteur Institute (Tehran, Iran). RPMI1640 culture medium, FBS, 0.25% trypsin-EDTA solution and phosphate buffered saline (PBS) tablets were obtained from Life Technologies Ltd. DMSO (dimethyl sulfoxide), penicillin, streptomycin and thymoquinone were obtained from Sigma-Aldrich. A549 cells were cultured in

25 cm^2 flasks in RPMI1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (100 units/ml penicillin and 100 µg/ml streptomycin). The cultured cells were grown at 37°C in humid air with 5% CO2.

MTT assay: cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Briefly, cells were cultured at a density of 1×10^4 in 96-well plates and incubated at 37°C with 5% CO2. The treatment was done with different concentrations of thymoquinone (0, 20, 40, 60, 80 and 100 µM) and the cells were incubated for 24 and 48 hours. Then, 10 µl (5 mg/ml) MTT was added to each well and the plate was incubated for 4 h at 37°C in the dark to form formazan by mitochondrial succinate dehydrogenase. The contents of the wells were replaced with 100 µL of DMSO and the absorbance was measured at 570 nm by Sunrise Tecan, Switzerland. The inhibition rate was calculated with the formula: Inhibitory Rates (IR)= 1- ($OD_{treatment}/OD_{control}$) × 100.

Lysate preparation: Cells were trypsinized and after centrifugation, cells were washed twice with cold PBS. Protease inhibitor cocktail + PBS was used to prepare cell suspension. Then the cell lysates were subjected to the freeze-defreeze cycle 3 times. The suspension was centrifuged at 12000 rpm for 15 minutes and the supernatant was collected in tubes and stored at -20°C.

Total Antioxidant Capacity (TAC): TAC was measured by manual FRAP method. This study evaluated the ability of sample antioxidant compounds to reduce Fe3±TPTZ complex to Fe2±TPTZ. The reaction mixture containing 300 µl of FARP reagent (300 mM acetate buffer, pH=3.6, 10 mM TPTZ and 20 mM iron chloride) and 10 µl of sample was poured into the wells of a 96-well plate and was incubated for 10 minutes in the dark at 37°C. The concentration of standards (1M $FeSO_4$) and samples was measured by a spectrophotometer (Bell, Italy) at a wavelength of 593 nm. Then, the concentration of each sample was determined based on the standard curve (22).

Total Oxidative Status (TOS): TOS was assessed using the ferrous oxidation−xylenol orange (FOX) assay based on the oxidation of ferrous ions to ferric ions, which results in the production of a Ferric-Xylenol Orange complex. The reaction mixture included 10 µl of sample and 190 µl of FOX reagent (250 µM ferrous ion, 150 µM Xylenol Orange, 100 mM sorbitol and 25 mM H_2SO_4 with pH=1.8) in a a 96-well plate. H_2O_2 and PBS were used as standards and blanks, respectively. Absorbance at 560 nm was read after 10 min of incubation at room temperature (23). Finally, the oxidative stress index (OSI) was defined as the percentage ratio of TOS to TAC.

GPx Enzyme Activity: Glutathione peroxidase (GPx) activity was determined using a ready-to-use Kiasyst kit (Tehran, Iran) based on the reduction of H_2O_2 by oxidation of glutathione, which acts as an electron donor. The enzyme converted GSH to GSSG, and the remaining GSH in the reaction medium can produce DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)) which has a yellow color and absorbs at 412 nm. Color production is inversely related to enzyme activity. Finally, GPx activity was reported as U/mg protein.

SOD enzyme activity: superoxide dismutase (SOD) activity was measured using a ready-to-use kit (Tehran, Iran). In this method, superoxide anion is used to convert resazurin into resorufin, and chromogen is produced. Finally, the absorbance of the chromogen was measured at 420 nm and the results were reported as U/mg protein.

CAT enzyme activity: Catalase activity was measured using the spectrophotometric method described by Hadwan and Abed (21). Briefly, 100 µL of sample was added and incubated with 1 mL of reagent (20 mM H_2O_2 in 50 mM sodium phosphate buffer, pH=7.4) at 37°C for 3 minutes. Then, 4 ml of ammonium molybdate (32.4 mM) was added to stop the reaction. The absorbance of the yellowish mixture can be measured at 374 nm. Catalase activity was calculated using the formula: Catalase Activity kU= 2.303/t × [log S0/S-M] × Vt/Vs.

The data were analyzed using SPSS version 16.0, and t-test was used to compare the differences between groups. One-way ANOVA and Tukey-Kramer post-hoc test were used to analyze the difference between groups. Values were displayed as Mean±SD and $p<0.05$ was considered significant.

Results

Genistein significantly decreased the survival of A549 lung cancer cells after 24 hours in a dose-dependent manner ($p<0.05$) (Figure 1). The IC50 dose of genistein on A549 cells was 40 µM, and the study was conducted by examining the effects of the IC50 dose and higher and lower doses (20 and 60 µM). Catalase and superoxide dismutase activity after 24-hour treatment with genistein in all three doses of 20, 40 and 60 µM decreased significantly compared to the untreated group ($p<0.05$). However, there was no significant difference between the treated groups (Figure 2-A and 2-B). Treatment with genistein significantly reduced glutathione peroxidase enzyme activity only at a dose of 60 µM ($p<0.05$) (Figure 2-C).

Our results showed that TOS was increased in cells exposed to genistein compared to the control group. Also, a significant difference was seen between the 60 µM dose group with 20 and 40 µM ($p<0.05$) (Figure 3-A), while TAC decreased in a dose-dependent manner. This decrease was significant at the dose of 60 µM ($p<0.05$) (Figure 3-B). Moreover, the OSI index shows an increase in oxidative stress conditions in all exposed groups (Figure 3-C).

The results related to pentraxin 4 protein show that a significant dose-dependent decrease in the amount of this protein is observed; there is a significant difference between 40 and 60 µM doses of genistein compared with 20 µM dose and control ($p<0.05$) (Figure 4).

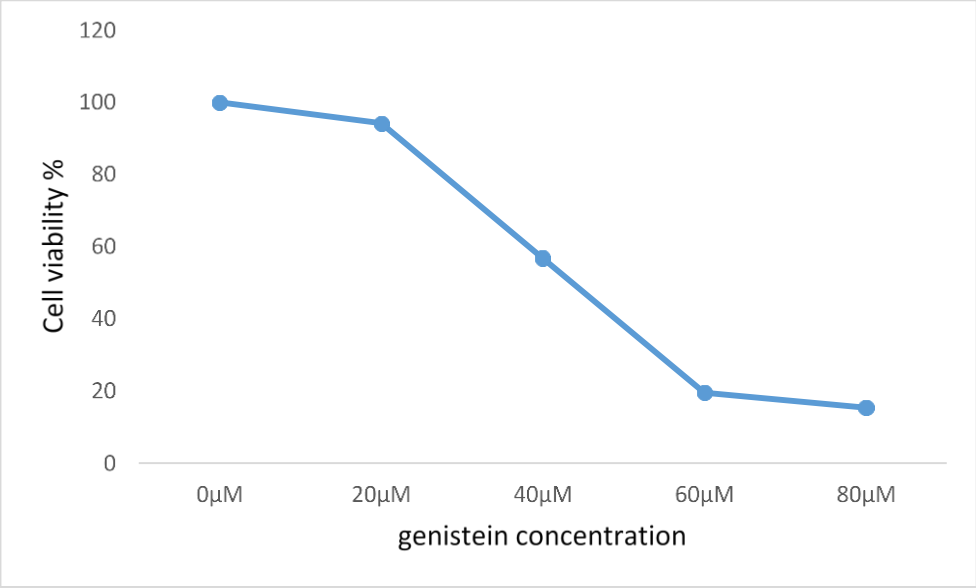

Figure 1. Viability of A549 lung cancer cells after 24-hour treatment with 0, 20, 40, 60 and 80 µM genistein

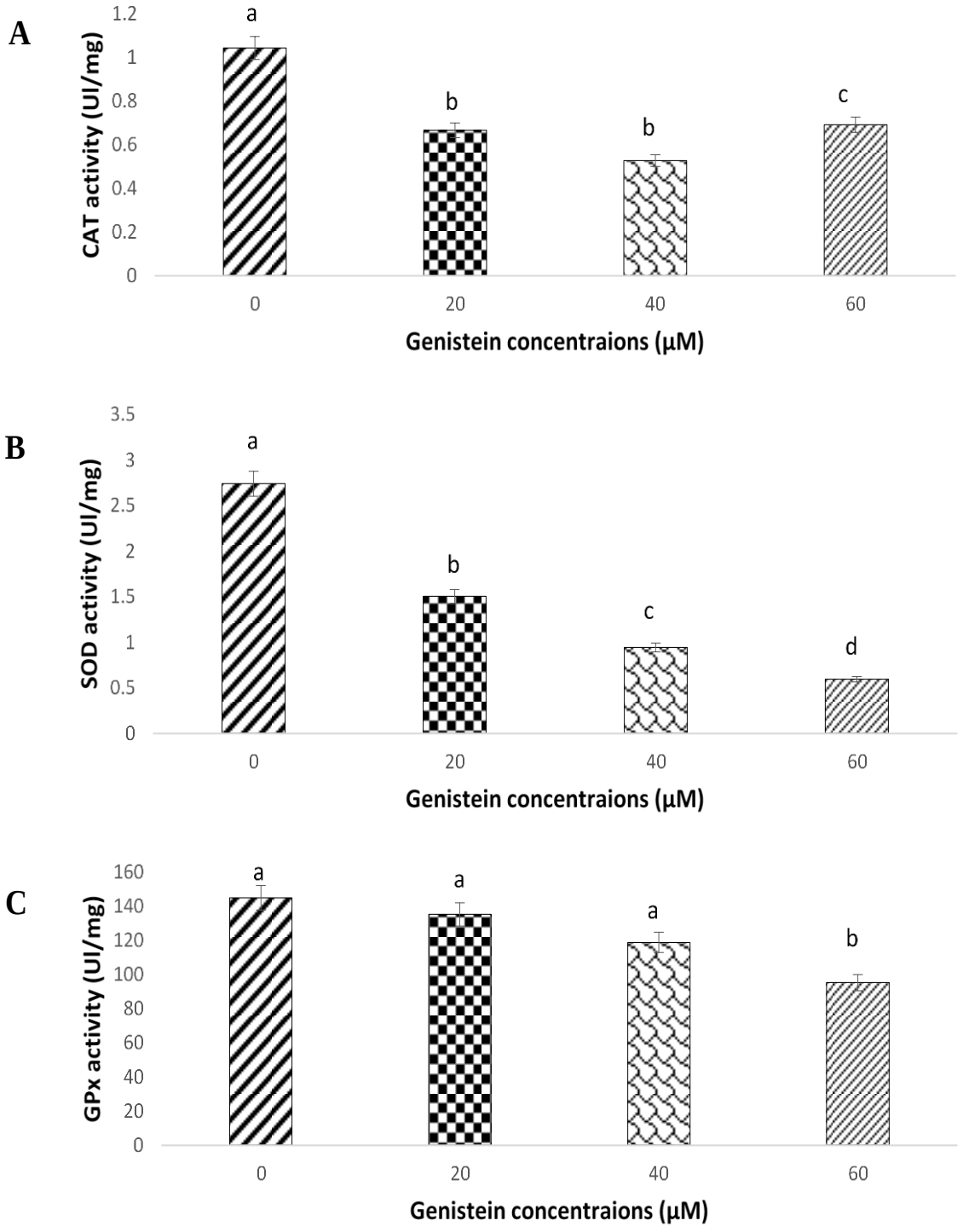

Figure 2. Measurement of antioxidant activities of enzymes CAT, SOD and GPx after 24 hours treatment with 20, 40 and 60 µM genistein. A) CAT, B) SOD, C) GPx. a: significant difference with the control group ($p<0.05$) and b: significant difference with the group treated with 20 and 40 µM doses

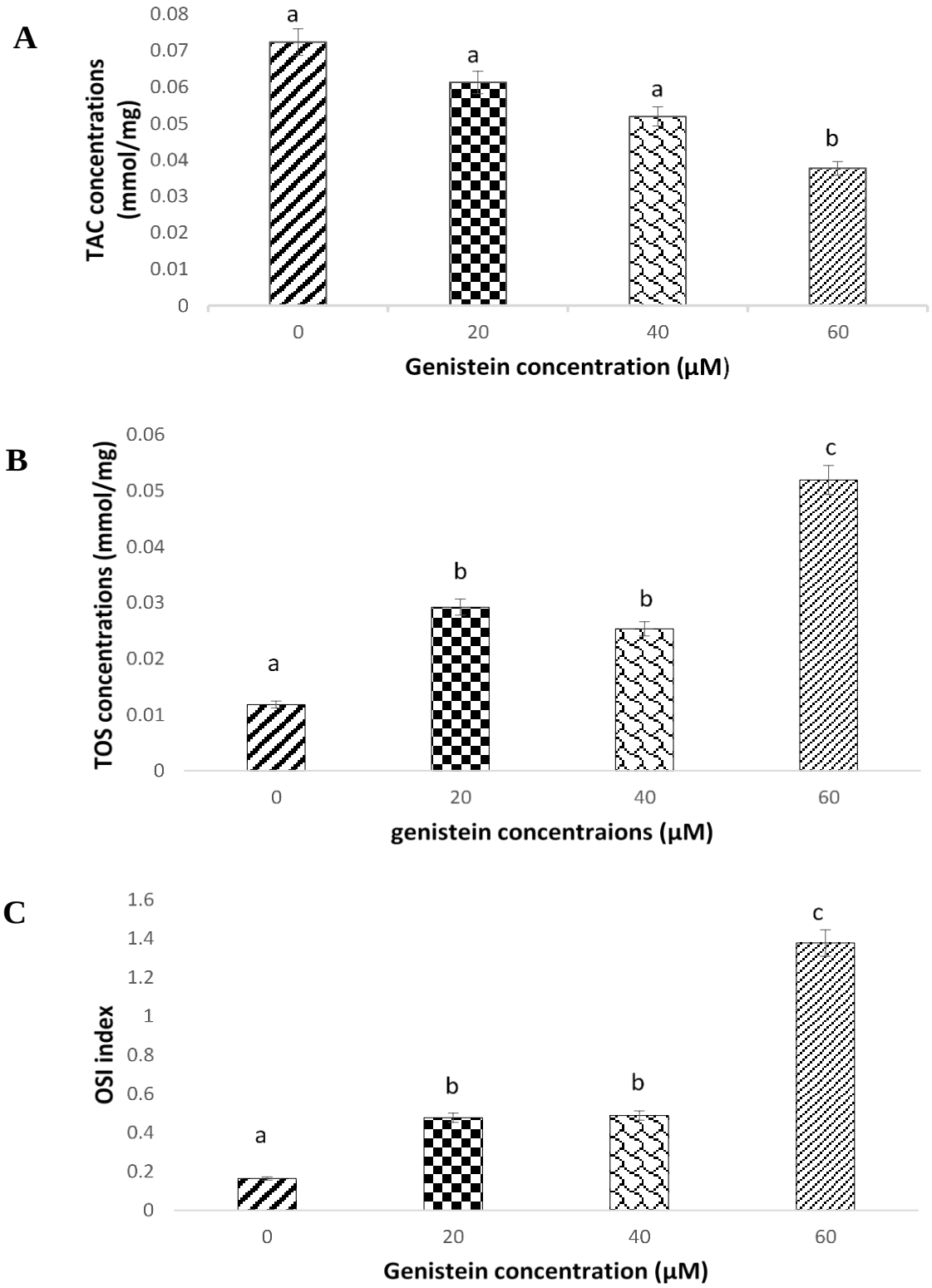

Figure 3. Measurement of TAC, TOS and OSI after 24-hour treatment with doses of 20, 40 and 60 µM genistein. **A) TOS, B) TAC, C) OSI. a: significant difference with the control group ($p<0.05$) and b: significant difference with the group treated with 20 and 40 µM doses**

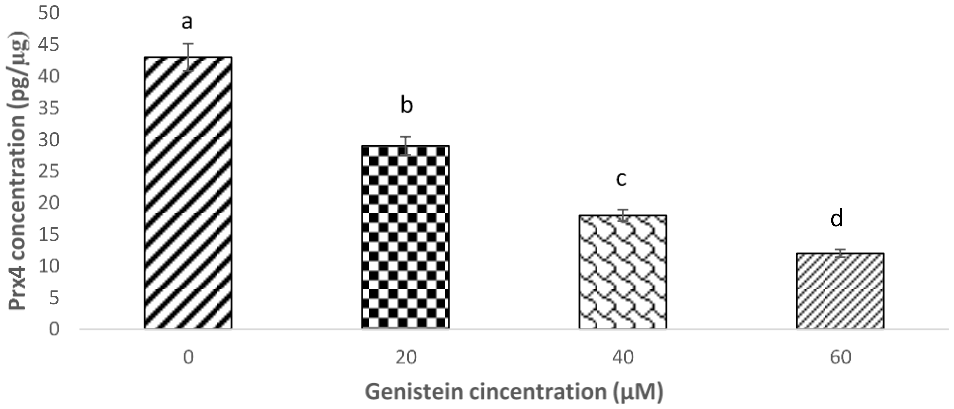

Figure 4. Measurement of pentraxin 4 after 24-hour treatment with 20, 40 and 60 µM doses of genistein. a: significant difference with the control group ($p<0.05$) and b: significant difference with the group treated with 20 and 40 µM doses

Discussion

Our results showed that exposure to genistein decreases cell viability and induces oxidative stress in A549 cell line, and by increasing TOS and decreasing GPx, SOD, CAT and TAC, it induces oxidative stress in A549 cells. Moreover, our results showed that genistein has pro-oxidative effects at low concentrations. The anticancer effect of genistein has been investigated in some cancers such as prostate (PCa), ovary and colon (DLD-1) (24-26). TOS and TAC are two reliable and accurate indicators that are mostly used to determine the state of oxidative stress (27, 28). In 2019, Zhang et al. investigated the effect of genistein on liver cancer HepG2 cell line and obtained results consistent with our results regarding oxidative stress. They reported that increasing the concentration of genistein increases the amount of reactive oxygen species in this cell line, which indicates the induction of oxidative stress by genistein (29). Also, the oxidative effect of genistein has been reported in some other cell lines such as Mia-PaCa2 (pancreatic cancer) and MDA-MB-231 (breast cancer) (30, 31). In contrast, the results by Raschke et al. indicate the antioxidant effect of genistein in prostate cancer. This difference in results could be due to the difference in cell lines (32).

SOD, GPX and CAT are the main antioxidant enzymes that play an important role in reducing oxygen radicals and cellular oxidative status. SOD converts superoxide radicals into molecular oxygen and hydrogen peroxide (H_2O_2). Finally, GPX and CAT remove the produced hydrogen peroxide to prevent its transformation into harmful substances such as hydroxyl radicals by the Fenton reaction (28). Our findings showed that the activities of SOD, GPX and CAT in genistein-treated cells were significantly reduced compared to untreated cells. The results of Prietsch et al., in line with our results, indicate a decrease in the expression of genes of antioxidant enzymes in MCF-7 cell line, such as SOD, when exposed to genistein (33). However, different studies are against our results and have shown increased activity of SOD, CAT and GPx in LNCaP, PC3 and HT29 lines (20, 34, 35). This study showed that treatment of A549 cell line with genistein causes oxidative stress in these cells and decreases the viability of cancer cells. It seems that the paradoxical effect of genistein probably depends on its concentration. The diversity of reports on genistein's pro-oxidant/anti-oxidant effects are due to its redox reactions. To further investigate the mechanism of action of this substance, more experiments are needed to measure ROS and cell apoptosis and investigate cancer and anticancer signaling pathways.

In this study, genistein induced oxidative stress in A549 lung cancer cells. Genistein increased TOS and decreased the activity of TAC, PRDX4, SOD, GPX and CAT with the greatest pro-oxidant effects. This study suggests genistein as a potential anticancer agent in A549 lung cancer cells.

Conflict of Interest: The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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