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Cytotoxic, genotoxic and apoptotic effects of naringenin-oxime relative to naringenin on normal and cancer cell lines

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ABSTRACT

Objective: To assess and compare the cytotoxic, genotoxic, apoptotic and reactive oxygen species (ROS) generating effects of naringenin (NG) and its new derived compound naringenin-oxime (NG-Ox) on MCF-7, HT-29, PC-12 cancer and L-929 normal cell lines.
Methods: The cells were incubated with different doses of NG-Ox and NG (50–1 000 μmol/L) for 24 h. The cell viability was assessed based on ATP cell viability assay. Intracellular accumulation of ROS was determined using the fluorescent probes 2′7′-dichlorodihydrofluorescin diacetate. Genotoxic effects were evaluated by alkaline single cell gel electrophoresis assay (comet assay) and, the apoptotic effect was evaluated by acridine orange staining at below the IC_{50} levels.
Results: Both NG-Ox and NG exhibited cytotoxic, genotoxic and apoptotic effects and resulted in increased ROS values in a dose-dependent manner. The effects were more pronounced on cancer cell lines. The cytotoxic, genotoxic and apoptotic effects of NG-Ox were higher than that of NG in all cell lines. Significant correlations were observed between cell viability, DNA damage, apoptosis and ROS, in all cell lines exposed to either NG-Ox or NG.
Conclusions: This study showed that both NG-Ox and NG possess cytotoxic, genotoxic and apoptotic activities through the production of ROS on cells, NG-Ox being the more effective one. Therefore, derived compound of NG might be used as antiproliferative agents for the treatment of cancer.

1. Introduction

Cancer chemoprevention is defined as the use of substances of natural origin, biological agents, synthetic, or chemical compounds to reverse, suppress or prevent carcinogenic progression of invasive cancer [1]. The main form of treatment at this point is chemotherapy, which consists of delivering drugs systemically so that they can reach and kill the tumor cells. But most of these drugs cause severe side effects in patients and need to be used at suboptimal levels. Therefore, studies have been focused on alternative chemopreventive agents such as some plant extracts, natural compounds obtained from plants or their derivatives. For example, flavonoids are found at high concentrations in citrus fruits in which the most common classes are flavanones, flavones, and flavonols [2,3]. These compounds exhibit a wide range of biological activities and positive health effects on mammalian cells, including anti-inflammatory, antiatherogenic, and anticancer [4]. Together with strong antioxidant activity, numerous studies have reported that flavonoids can show cytotoxic and apoptotic effects on various cancer cell lines [5,6].

Generally cytotoxic and antitumor agents are non-selective and kill normal proliferating cells. Identification of active cancer specific compounds remains a thrust area in drug screening and drug discovery mechanisms. Much emphasis has been

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placed on discovering new compounds that target tumor cells more efficiently and selectively with minimal toxic effects on normal cells [7,8]. Naringenin (NG) is a member of flavonoids, which is also abundant in citrus fruits. It has been reported to have different biological activities such as antioxidant, free radical scavenging, anti-inflammatory and immunomodulatory effects [9–11]. In addition to these potentials, NG has also been reported to induce cytotoxic and apoptotic activities in various cancer cell lines [12–14]. Although, the molecular mechanisms of its antiproliferative and apoptotic effects have not been cleared [15], increasing evidence have supported that the increase of reactive oxygen species (ROS) generation contributes to the treatment of cancer cells [16]. Khan *et al.* [17] demonstrated that the cytotoxic effect of NG is because of its pro-oxidant activity.

To seek for the derivatives of this compound with possible higher activity Turkkan *et al.* [18] synthesized, characterized and investigated the antioxidant properties of a new NG-derivatized compound called NG-Oxime (NG-Ox), and demonstrated that antioxidant and antigenotoxic properties of NG were significantly enhanced in its Ox form. Kocyigit *et al.* [19] demonstrated that both NG and NG-Ox are able to protect cells against oxidative damage and NG-Ox is more effective than NG.

Özyürek *et al.* [20] also showed it's antiproliferative activity. However, there is no available report comparing cytotoxic, genotoxic, apoptotic potentials of NG-Ox relative to NG. The aim of the present study was to evaluate the cytotoxic, genotoxic, apoptotic and ROS generating effects of NG-Ox relative to NG in several cancer and normal cell lines to seek for a hope in cancer treatment.

2. Materials and methods

2.1. Chemicals

Naringenin [(±)-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], heat-inactivated fetal calf serum, Dulbecco's modified Eagle medium (DMEM), 2′7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA), pencillin-streptomycin and ethidium bromide (EB) were purchased from Sigma–Aldrich (Seelze, Germany). NG-Ox [(±)-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one oxime] was synthesized and characterized by Turkkan *et al.* [18]. All other reagents used were of analytical grade unless otherwise stated.

2.2. Samples

Both NG and NG-Ox were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (40 mmol/L). The stock solution was diluted with DMEM (contains no fetal bovine serum). The final concentration of DMSO in the NG and NG-Ox solution was < 1%. Prior to start of experiments, we confirmed that this level of DMSO as well as the serum free media did not induce any DNA damages in the cells. Other reagents were prepared as fresh before each experiment.

2.3. Cell culture and maintenance

L-929 cells (as a standard cell line originated from mouse fibroblast cells) and human colorectal adenocarcinoma cell line (HT-29), were obtained from American Type Cell Culture Collection. Rat pheochromocytoma cell line (PC-12) and human breast cancer cell line (MCF-7) were kindly supplied as gifts from Prof. Dr. S. Ismet Delioglu Gurhan (Bioengineering Department, University of Ege, Turkey). All cells were cultured in DMEM equilibrated with 5% CO_2 atmosphere at 37 °C. The medium was supplemented with 10% fetal calf serum, 100 IU/mL of penicillin and 100 ng/mL of streptomycin. The number of viable cells was estimated by trypan blue exclusion test.

2.4. Cytotoxicity assay

Cytotoxic activities of NG-Ox and NG on HT-29, PC-12, MCF-7 and L-929 cells were determined by ATP levels using a luminescence test (Cell-Titer-Glo luminescent cell viability assay, Promega). Cells were seeded onto 96-well plates at a density of 5×10^3 cells per well and incubated overnight at 37 °C in 5% CO_2. The medium was then replaced with fresh complete medium containing various concentrations of NG-Ox and NG (50–1 000 μmol/L). Control cells were treated with 1% DMSO. All the cells were incubated in a humidified 5% CO_2 and 95% O_2 at 37 °C for 24 h. Then, the cells were rinsed with the culture medium and tested for ATP. Each of the samples was supplemented with 100 μL of the prepared reagent (CellTiter-Glo luminescent cell viability assay, Promega), mixed for 2 min and incubated for 10 min at room temperature. The results were read using a luminometer (Varioskan Flash Multimode Reader, Thermo, Waltham, MA). The light emitted in the presence of ATP was quantitated in relative light units. The intensity of emitted light quants was directly related to ATP content in the tested sample. The cell viability was expressed as the percentage compared with the negative control group designated as 100%. IC_{50} values were calculated from the concentration–response curves by non-linear regression analysis. All experiments were repeated three times and standard deviation was within 5%.

2.5. Measurement of ROS generation

Generation of ROS was assessed by using a cell-permeable fluorescent signal H_2DCF-DA as an indicator for ROS [21,22]. As described previously, H_2DCF-DA is oxidized to a highly green fluorescent 2′7′-dichlorofluorescein (DCF) by the generation of ROS. Cancer cell lines were pretreated with various concentrations of NG and NG-Ox for 24 h. After 24 h incubation period, the cells were washed with cold phosphate buffer solution (PBS) and incubated with 100 mmol/L H_2DCF-DA for another 30 min at 37 °C. DCF fluorescence intensity was measured using the fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, MA) at excitation/emission = 488/525 nm. The estimations were carried out thrice in triplicate, ensuring each time that the number of cells per treatment group were the same to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.

2.6. Genotoxic activity assays

Genotoxic effects of NG and NG-Ox on HT-29, PC-12, MCF-7 and L-929 cell lines were evaluated by using alkaline single cell gel electrophoresis assay (comet assay) according to Singh *et al.* [23] with slight modification. To determine the

genotoxic potential of NG-Ox and NG, three different cells were seeded onto 6-well cell culture plates (approximately 2×10^5 cells per well) with cell culture medium and incubated at 37 °C in 5% CO_2 for 24 h for cell establishment. After 24 h, below IC_{50} concentrations of NG and NG-Ox (50–600 μmol/L in 1% DMSO) were added to the cells and incubated for another 24 h at 37 °C. DMSO (1%) was used as a negative control and 50 μmol/L H_2O_2 was used as a positive control. After incubation, the cells were washed with PBS, harvested using trypsin/ethylene diamine tetraacetic acid (EDTA) and collected for centrifugation at 1 750 r/min for 5 min at 4 °C. The supernatant was discharged and the cell density was adjusted to 2×10^5 cells/mL using cold PBS. Re-suspended cells (10 μL) were placed into centrifuge tubes for the comet assay as described below. All experiments were repeated in triplicate.

Cell suspensions (10 μL) was mixed with 90 μL of 0.6% low melting agarose and added to the slides pre-coated with 1% normal melting agarose [24]. After solidification of the agarose, the slides were immersed in lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4 °C. After removing the slides from lysis solution, they were washed with cold PBS and placed in a horizontal electrophoresis tank side by side. DNA was allowed to unwind for 40 min in freshly prepared alkaline electrophoresis buffer containing 300 mmol/L NaOH and 10 mmol/L Na_2EDTA (pH 13.0). After unwinding, electrophoresis was run at 300 mA for 25 min at 4 °C under minimal illumination to prevent further DNA damage. The slides were washed three times with a neutralization buffer (0.4 mol/L Tris, pH 7.5) for 5 min at 4 °C and then treated with ethanol for another 5 min before staining. Dried microscope slides were stained with EB (2 μg/mL in distilled H_2O; 70 μL/slide) covered with a coverslip and analyzed using a fluorescence microscope (Leica DM 1000, Solms, Germany) at a 200 magnification with epiflourescence equipped with a rhodamine filter (with an excitation wavelength of 546 nm; and a barrier of 580 nm). A hundred cells were randomly scored by eye in each sample on a scale of 0–4 based on fluorescence beyond the nucleus. The scale used was as follows: 0, no tail; 1, comet tail > half the width of the nucleus; 2, comet tail equal to the width of the nucleus; 3, comet tail longer than the nucleus and 4, comet > twice the width of the nucleus. Scoring cells in this manner have been shown to be as accurate and precise as using computerized image analysis. The individual scoring of the slides was blind, using coded slides. The visual score for each class was calculated by multiplying the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score characterizing the degree of DNA damage in the entire study groups was the sum of the scores in the five comet classes. Thus, the total visual score could range from 0 (all undamaged) to 400 (all maximally damaged) arbitrary units (AU), as reported by Collins *et al.* [25]. This method of measurement was proved to be valid and up-to-date [26]. All of the procedures were completed with the same biochemistry staff and DNA damage was detected using a single observer that was not aware of the subject's status. Cell viability measured with trypan blue exclusion test was above 95% for all treatments. Results of the triplicated tests are expressed in terms of arbitrary units recorded. All experiments were repeated in triplicate.

2.7. Morphological evaluation by fluorescence microscopy

Morphological changes in cells were studied by acridine orange/ethidium bromide (AO/EB) double staining as described by McGahon *et al.* [27]. Using this technique, the cells undergoing apoptosis are distinguished from the viable cells by the morphological changes of apoptotic nuclei. Ethidium bromide and acridine orange are DNA intercalating dyes. Acridine orange is taken up by both viable and dead cells and stains double-stranded and single-stranded nucleic acids. When acridine orange diffuses into double-stranded DNA, it emits green fluorescence upon excitation at 480–490 nm from viable cells [28]. Ethidium bromide is taken up by dead cells and stains DNA orange. Briefly, the cells were cultured in six-well plates (2×10^5 cells/well) and incubated overnight. Then, the cells were treated with NG and NG-Ox under doses of IC_{50} determined by cytotoxicity assay for 24 h at 37 °C. DMSO (1%) was used as a negative control. The cells were then harvested and washed twice with PBS. Finally, AO/EB solution was added to the cell suspension and the nuclear morphology was evaluated by fluorescence microscopy (Leica DM 1000, Solms, Germany). Multiple photos were taken at randomly-selected areas and a minimum of 100 cells were counted. According to the method, the live cells have normal green nuclei, apoptotic cells have green nuclei with fragmented chromatin, and dead cells have orange/red nuclei. Tests were done in triplicate.

2.8. Statistical analysis

The results are presented as the mean ± SD of tree replicates. Data in all experiments were analyzed for statistical significance using analyses of variance (One-way ANOVA). The P value < 0.05 was considered as statistically significant. IC_{50} values of NG and NG-Ox over the cell lines were calculated by nonlinear regression analysis. Associations between ROS generation and cell viability parameters were analyzed by Pearson correlation coefficient. All statistical analyses were performed using SPSS package program for Windows (Version 20, Chicago, IL).

3. Results

3.1. Cytotoxicity of NG and NG-Ox

The effects of NG and NG-Ox on the viability of HT-29, PC-12, MCF-7 cancer cells and L-929 normal cells were determined via bioluminometric ATP cell viability assay. Our findings showed that NG-Ox and NG resulted in greater cellular death in HT-29, MCF-7 and PC-12 cancer cells than that of L-929 cells. The percentage of anti-proliferative activity progressively increased in a concentration dependent manner and, NG-Ox was more effective than NG in all cell lines (Figure 1).

While IC_{50} of NG-Ox for MCF-7, HT-29, PC-12 and L-929 cells ranged between 470 and 790 μmol/L, NG was less effective with the IC_{50} values of 780–880 μmol/L for MCF-7, HT-29 and PC-12 cells. IC_{50} values of NG for L-929 cell lines could not be determined since the value was out of range. IC_{50} values for NG-Ox and NG for the cell lines are given in Table 1.

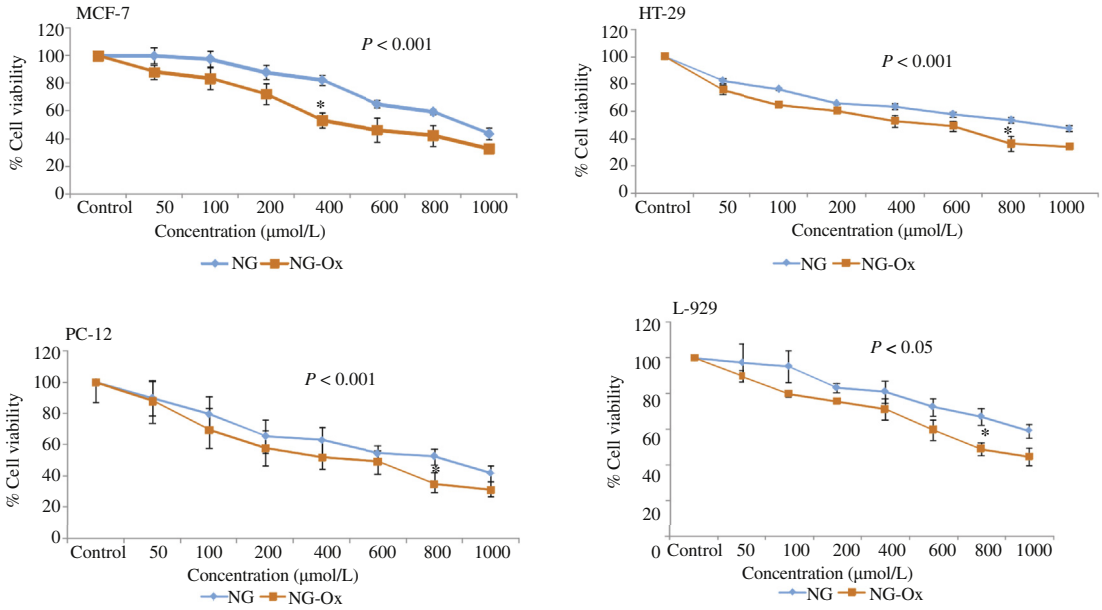

Figure 1. Effects of NG and NG-Ox on the viability of HT-29, PC-12, MCF-7 cancer cells and L-929 normal cells.
Cells treated with NG and NG-Ox at different concentrations (50–1 000 μmol/L) for 24 h. The results were expressed as % viability.

Table 1

The IC_{50} values of NG and NG-Ox for MCF-7, HT-29, PC-12 and L-929 cells. μmol/L.

Cell lines	IC_{50} value	
	NG	NG-Ox
MCF-7 (Human breast cancer cells)	780	470
HT-29 (Human colon cancer cells)	870	560
PC-12 (Rat pheochromocytoma cells)	880	575
L-929 (Mouse fibroblast cell line)	–	790

Values are the mean of three measurements.

3.2. ROS generation ability of NG and NG-Ox

It has been entrusted that different ROS species are produced endogenously in many cell types in diverse cellular processes [29]. It has also been proposed that ROS are active cellular signaling molecules [30], and have unabated role in the induction of apoptosis [31]. In this context to check whether ROS production is associated with NG and NG-Ox induced apoptosis in cancer and normal cells, we evaluated intracellular ROS generation by fluorimetry using H_2DCF-DA as a probe. We have observed that NG and NG-Ox could efficiently induce ROS generation in all cancer cells in a concentration dependent manner and, NG-Ox was more effective than that of NG (Figure 2).

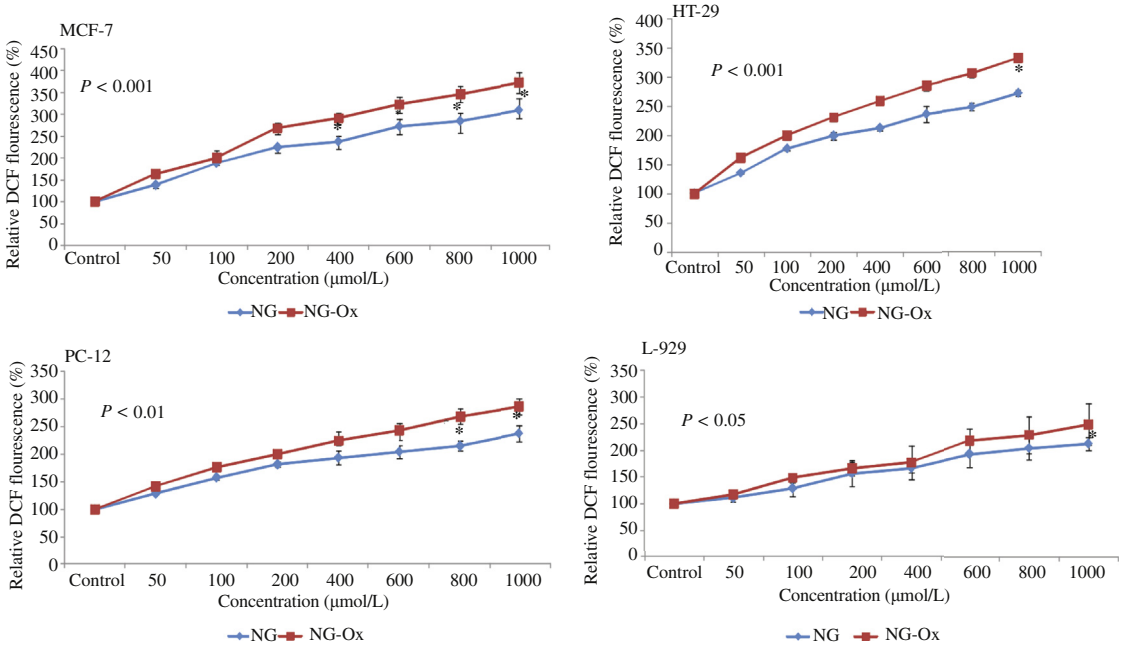

Figure 2. ROS generating effect of NG-Ox and NG on MCF-7, HT-29, PC-12, cancer cells and L-929 normal cells.
NG and NG-Ox induced ROS generation was measured using fluorescent dye DCF-DA by fluorimetry. Data presented were mean ± SD (n = 3).

There was a significant negative relationship between ROS generating activity and cell viability in all cell lines ($P < 0.001$).

3.3. Genotoxic activity

The genotoxic effects of the NG-Ox on cancer and normal cell lines were also investigated using comet assay (Figure 3).

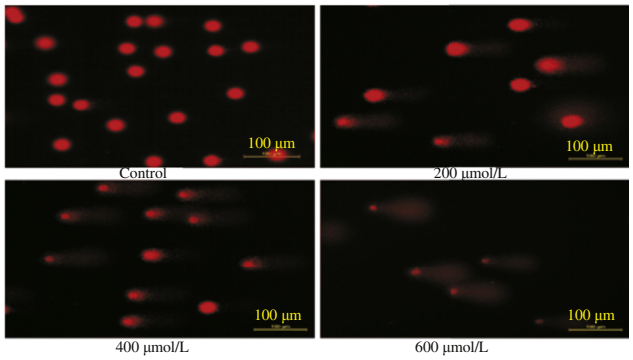

Figure 3. Genotoxic effect of NG-Ox (200–600 μmol/L) on MCF-7 cancer cells after 24 h incubation.
Comet formation pattern showed that NG-Ox induces DNA damage in a dose dependent manner.

For the evaluation of genotoxicity, the cells were treated with NG-Ox and NG at the under concentrations of their IC_{50}. The results of genotoxic activity of NG-Ox and NG are presented in Figure 4. As seen in Figure 4, both NG-Ox and NG significantly induced DNA damages in MCF-7, HT-29, PC-12 and L-929 cell lines (above 50, 100, 200, 400 μmol/L, respectively) and, NG-Ox was more effective than NG in all cell lines.

3.4. Morphological evaluation

For the evaluation of apoptosis, the cells were treated with NG and NG-Ox at various concentrations (50–600 μmol/L) arranged according to their IC_{50} values. After 24 h incubation, AO/EB staining was used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells were viewed under a fluorescence microscope, and the images of apoptotic and control cells after AO/EB are presented in Figure 5.

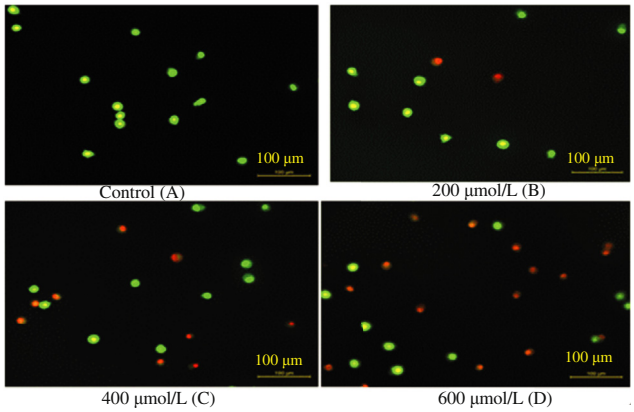

Figure 5. Different morphological patterns of apoptosis induced by NG-Ox determined by AO/EB double staining.
Cells were treated with NG-Ox (50–600 μmol/L) for 24 h and, subjected to AO/EB staining. A: Control showing intact nuclei with green fluorescence. B–D: Cells showing NG-Ox-induced increased membrane bound apoptotic bodies with yellowish orange nuclei in a dose depended manner. All experiments were repeated three times.

The apoptotic cells had orange particles in their nuclei whereas the viable cells were observed green. The results indicated that exposure of the cells below IC_{50} doses of NG-Ox caused generally more apoptotic activity than those of NG in

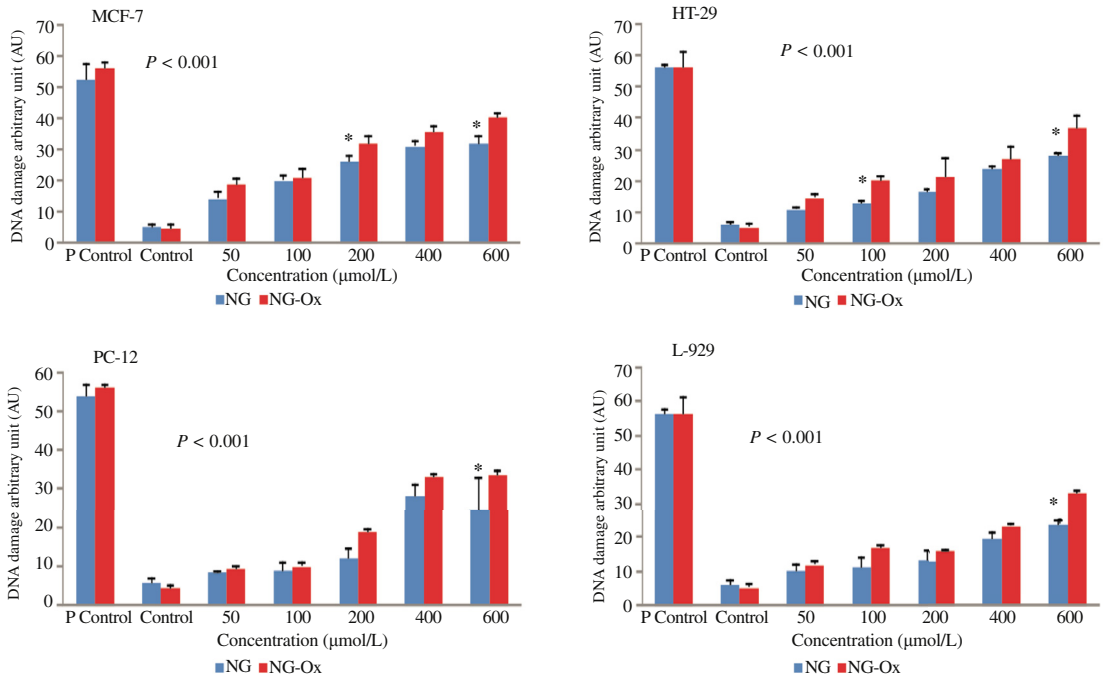

Figure 4. The comet assay results of NG and NG-Ox on HT-29, MCF-7, PC-12 and L-929 cell lines.
Values are shown as mean ± SD which are three separate experiments performed in triplicate. P Control: Positive control.

all cancer cell lines. As seen in Figure 6, both NG-Ox and NG significantly induced apoptosis in MCF-7, HT-29, PC-12 and L-929 cell lines and NG-Ox was more effective than NG in all cancer cells (Figure 6).

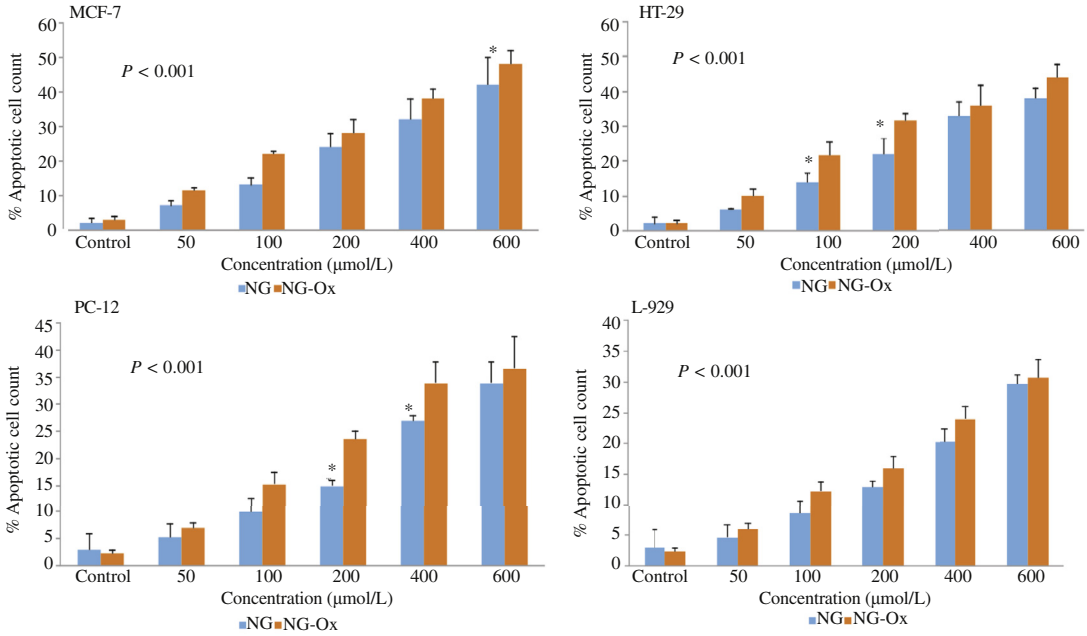

Figure 6. Apoptotic activity of NG and NG-Ox on HT-29, MCF-7, PC-12 and L-929 cell lines.
Cells were treated with different concentrations of NG and NG-Ox (50–600 μmol/L) 24 h, AO/EB double staining and measured by fluorimetry. Data presented were mean ± SD (n = 3).

There were positive correlations between genotoxicity, apoptosis and ROS levels ($P < 0.001$) and a negative relationship between cell viability and ROS generating activity ($P < 0.001$) exposed to either NG-Ox or NG in all cell lines.

4. Discussion

Natural products or their derivatives have been demonstrated to have significant anticancer potentials due to their ability to inhibit tumor growth, angiogenesis and metastasis without many side effects [32–35]. Much of these activities come from flavonoids, which are principal components of many phytochemicals and demonstrate the capacity to inactivate carcinogens, inhibit angiogenesis, and promote healthy cell proliferation and apoptosis. In the present study, we demonstrated that treatment with NG-Ox showed more cytotoxic activity on several cancer and normal cells relative to NG in a dose-dependent manner. Since the cell death activity of NG-Ox was higher, we further evaluated to reveal the mechanism of its cytotoxicity. For this purpose, we determined genotoxic, apoptotic and ROS generating activities of NG-Ox and NG.

The possible antiproliferative effects of the NG-Ox and NG were evaluated using ATP cell viability assay and we also evaluated IC_{50} values of these compounds. ATP is among the most sensitive luminometric tests to analyze cell culture viability. We observed that NG-Ox and NG resulted in great cellular death in HT-29, MCF-7 and PC-12 cancer cells and the percentage of antiproliferative activity progressively increased in a dose-dependent manner. Several *in vitro* studies also demonstrated that NG had antiproliferative effects in cancer cell lines. For example, Kanno *et al.* [36] found that NG caused cell death via apoptosis in various human cancer cell lines and had an inhibitory effect on tumor growth in sarcoma S-180-implanted mice. Similarly, Park *et al.* [15] showed that NG significantly induced antiproliferative activity via apoptosis in human leukemia THP-1 cells. However, the only report on cytotoxicity of NG-Ox is made by Özyürek *et al.* [20]. However, there is no available report comparing antiproliferative activity between NG-Ox and NG.

Flavonoids are universally recognized antioxidants which can protect the cell from the oxidative stress, *i.e.*, neutralize the damaging effect of ROS. NG and NG-Ox have been well-known as antioxidant agents [18,20]. However, at high concentrations, flavonoids and other polyphenols can show cytotoxic effects [37]. The mechanism of dual protective-destructive behavior of flavonoids has not been elucidated yet. There are several mechanisms offered for the cytotoxicity of flavonoids including the inhibition of topoisomerases [38], kinases [39] and their pro-oxidant action [40]. It is highly possible that the pro-oxidant effect is responsible for the selective antiproliferative activity of these compounds and ROS are key signaling molecules to modulate cell death [41]. Accumulating evidence indicates that cancer cells produce high levels of ROS that lead to a state of increased basal oxidative stress. The increased production of ROS in cancer cells was observed in *in vitro* and *in vivo* studies [42]. It has been found that NG had pro-oxidant potential [43]. Ahamad *et al.* [44] demonstrated that NG leads to cell death in cancer cells via inducing ROS generation. We therefore investigated the effectiveness of NG and NG-Ox in generation of ROS. We found that exposure of cancer cells with NG and NG-Ox dramatically enhanced generation of intracellular ROS at different levels in a dose dependent manner and

NG-Ox is more effective than that of NG in all cell lines. A negative significant correlation was evident between cell viability and ROS generating activity in all cancer cells. These findings demonstrated that both endogenous and exogenous ROS generation might induce killing of cancer cells with a synergistic effect [45].

ROS are constantly generated and eliminated in the biological system, and play important roles in a variety of normal biochemical functions and abnormal pathological processes. Consequently, humans have evolved antioxidant defense systems that limit their production. Generation of oxidants plays a vital role to initiate the cellular apoptotic cascade by disturbing the balance between cellular signals for survival and suicide. Growing evidence suggests that cancer cells exhibit increased intrinsic ROS, due in part to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction [46]. We hypothesize that different metabolic activity of cancer cells result in different levels of ROS generation and, there is a close relationship between ROS generating potentials and cytotoxic activity of cancer cells. It has also been reported that higher basal levels of ROS in leukemic cells are more sensitive to pro-oxidants as compared to their normal counterparts [47]. Taken together, NG acting as pro-oxidant, effectively raised the cell's oxidative status beyond a threshold limit inducing apoptosis in leukemic cells.

Increased ROS generating and cytotoxic activity of NG-Ox compared to NG could be related to its molecular shape. Because, pro-oxidant activity is thought to be directly proportional to the total number of hydroxyl groups in a flavonoid molecule [48]. Series of mono and dihydroxy-flavonoids exhibited detectable pro-oxidant activity, while multiple hydroxyl groups, especially in the B-ring, significantly increased production of hydroxyl radicals in Fenton reaction [49]. As illustrated in Figure 1, NG has three OH groups while NG-Ox carries four in the ring B.

In this study, we measured genotoxic effects of NG and NG-Ox on cancer cell lines with alkaline comet assay, using non-cytotoxic concentrations of these two compounds. The comet assay is a sensitive method for detecting DNA strand breakage at the level of an individual cell. We observed that low concentrations of NG and NG-Ox caused no genotoxicity while the concentrations above 50 μmol/L showed genotoxic activities in MCF-7 cells. Previous studies have demonstrated that DNA damage increases with the increasing concentration of NG together with other flavonoids in lymphocyte cells. Similarly, it has been shown that NG and Morin can induce peroxidation in nuclear membrane lipids along with the increase in DNA strand breakage. Unexpected genotoxic behavior of these compounds could explain, in part, by their pro-oxidant activity [50–53]. It has been revealed that pro-oxidant compounds increase with the levels of H_2O_2 [54]. The H_2O_2 can kill any living cells and abort their development at any stage and results in DNA damage by the hydroxyl radical generated via Fenton reaction.

Since the main goal of our study was to obtain agents that promote or induce apoptosis, NG-Ox and NG with low IC_{50} against cancer and normal cells were selected to determine cell death mechanism (apoptosis or necrosis). Our results demonstrated that both NG-Ox and NG can show higher apoptotic activity on cancer cells. NG-Ox was more effective than NG in all cell types.

Apoptotic, necrotic and live cells can be distinguished using AO/EB staining. AO can penetrate living and dead cells and emits green fluorescence as a result of intercalation into double-stranded DNA while EB can only penetrate dead cells and emits red fluorescence after intercalation with DNA [55]. Therefore, fluorescent DNA binding dyes AO and EB were used to detect the apoptotic and necrotic cells (Figure 5). In this figure, living cells are distinguished with a normal green nucleus, apoptotic cells show orange-stained nuclei with chromatin condensation or fragmentations, while necrotic cells are clearly observed with uniformly orange-stained cell nuclei with no condensed chromatin. Analysis of the AO/EB staining results revealed that NG-Ox and NG treated cancer cells clearly exhibited late-stage apoptotic events (chromatin condensation and nuclear fragmentation) with a significant decrease in cell viability. It has been demonstrated that NG and its synthetic derivatives induced apoptosis in cancer cell lines in different pathways [50–53]. Apoptosis refers to programmed cell death. In the process of apoptosis, there are changes in the morphology of the cells, such as chromatin condensation and a reduction in cell volume, associated with fragmentation of DNA into nucleosomal size fragments of 180–200 bp or multiples thereof in such systems apoptotic bodies are formed [56]. Induction of apoptosis has been considered to be the major mechanism of anticancer drug discovery, and the development of anti-cancer drugs that inhibit abnormal cancer cell proliferation and induce cell death through apoptosis is a fundamental objective of cancer research [57,58].

The present *in vitro* study demonstrated that both NG and its new derivatized compound NG-Ox have more cytotoxic, genotoxic and apoptotic activity through the production of ROS on cancer cells than in normal cells and NG-Ox was more effective than NG at equal doses. Therefore, derivatized compound of NG might be used as antiproliferative agents for the treatment of cancer. Since our data is based on *in vitro* experiments, further *in vivo* studies are required to consolidate the genotoxic and antiproliferative properties of NG and NG-Ox.

Conflict of interest statement

We declare that we have no conflict of interest.

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