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Anti-Oxidative Effect of Genistein and Mangiferin on Sodium Fluoride Induced Oxidative Insult of Renal Cells: A Comparative Study

Pritam Sadhukhan, Sukanya Saha, Parames C Sil

Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M, Kolkata-700054, India

Corresponding author: Parames C Sil

parames@jcbose.ac.in
parames_95@yahoo.co.in

Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M, Calcutta-700054, West Bengal, India.

Tel: 9133-25693243
Fax: 9133-2355-3886

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Abstract

Background: Sodium fluoride (NaF) is a well-known environmental pollutant prevalently found in drinking water and is also used for the treatment of dental problems. It promotes oxidative stress and disrupts tissue homeostasis in the vital organs of the body including kidney and liver. Two antioxidants, namely mangiferin and genistein, have been reported to exert protective effect against NaF-induced oxidative insult. These two polyphenols are known to modulate intracellular gene expressions under different pathophysiological states.

Methods and findings: The present study was designed to investigate the attenuative role of mangiferin and genistein against NaF induced cytotoxicity on normal kidney epithelial (NKE) cells. Several free radical scavenging assays indicate that both the polyphenolic compounds are effective in scavenging free radicals in cell free system. Further, dose and time dependent studies revealed that 500 µM NaF exposure for 16 hours optimally induced 50% cell death. Along with these data, several other assays investigating FRAP LDH leakage, lipid peroxidation, apoptosis, ROS generation, endogenous antioxidant system, mitochondrial membrane potential, cell cycle and immunoblot analysis indicated that NaF disrupted the intracellular antioxidant potential of the renal cells. Pre-treatment with both mangiferin and genistein, however, exclusively prevented the induction of cytotoxicity induced by NaF at a dose of 5 and 10 µM respectively.

Conclusion: This study is expected to help in understanding the molecular toxicity induced by NaF and the protective role of mangiferin and genistein. The antioxidative and gene modulatory role of mangiferin and genistein were found to be effective in NaF induced oxidative stress in renal cells.

Keywords: Sodium fluoride; Oxidative stress; Antioxidants; Mangiferin; Genistein; Apoptosis

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Introduction

Sodium fluoride (NaF) is widely used in dental treatments to prevent cavity formation and provides resistance to decay due to acid and bacteria [1, 2]. The use of sodium fluoride is harmless and rather healthy up to 20 mg per day but it causes severe chronic and acute organ pathophysiology upon sustained use in higher amounts. For a person of 50 kg bodyweight, approximately, only 3 g of elemental fluoride is considered to be lethal. Apart from the medical use, elemental fluoride is widely used for different industrial purposes and is commonly present (in the form of different compounds) in drinking water, daily food, toothpaste, mouthwash and other dental medicine used to prevent tooth decay [3]. In case of prolonged exposure to fluoride, severe progressive disorder in skeletal muscle system and teeth is observed and this degeneration is often collectively called as fluorosis [4]. Excess intake of fluoride (e.g., sodium fluoride) also causes degeneration in the different parts of the central and

peripheral nervous system [5]. Current scientific reports suggest that acute and chronic fluoride exposure in experimental animals can cause severe damage not only in the myocardial tissue [6], but it also has the potential to perturb the normal homeostasis in different vital organs of a mammalian system including the liver, kidney and stomach [7].

Along with all the above mentioned ill effects of exposure, the accumulation of NaF induces intracellular oxidative stress [8]. Exogenous oxidative insult causes an elevation in the intracellular ROS level [9, 10]. Due to the oxidative environment, it disrupts the intracellular homeostasis between the endogenous free radical and antioxidant defence mechanisms of the body [11, 12]. Low level of intracellular ROS is critical for normal cellular homeostasis but at higher level it can reversibly impair different cellular functions and its further increase can cause irreversible cellular dysfunctions, formation of tumours and even induce cellular death [13]. Researches around the globe suggest that oxidative stress is one of the major culprits in renal dysfunctions too [14, 15]. So, it will be significant to develop an in vitro nephropathic model to study the fluoride induced oxidative stress mechanism in detail.

Numerous natural compounds are identified till date which is very much effective in neutralising the intracellular effect of exogenous oxidative insult [16-20]. Among them mangiferin [21] and genistein [22] are the two isoflvonoid compounds, consisting of several -OH groups which make them efficient free radical scavenger [23]. Scientific reports published from our and other laboratories around the globe suggest that these two pholyphenolic compounds are pleiotropic in nature, showing multiple beneficial effects on several pathophysiological conditions [24, 25]. Mangiferin is prevalently found in the mango plant whereas genistein is found in the plants from the Fabaceae family [26]. Mangiferin and genistein are extracted and purified from the bark of the mango plant and soybeans respectively. The study on the role of these two prophylactic natural compounds is very limited in sodium fluoride induced nephrotoxicity. We, therefore, designed our study to investigate the nature of toxicity induced by NaF and possible attenuative role of these antioxidantive small molecules in normal kidney epithelial cell (NKE cells).

In the present study the free radical scavenging activities of both the polyphenolic antioxidant molecules, mangiferin and genistein were first compared by performing several assays in cell free conditions. Followed by this, cellular toxicity caused by sodium fluoride in the NKE cells was evaluated by performing dose and time dependent MTT cell viability assay. Finally, the antioxidant status and cytoprotective effect of these phytochemicals (mangiferin and genistein) were evaluated by various experiments such as evaluating LDH leakage, lipid peroxidation end products, protein carbonylation, assessment of antioxidant enzymes, intracellular ROS level, mitochondrial membrane potential, cell cycle analysis and activities of some key regulatory proteins involved in cell death.

Materials and Method

Materials and reagent

Sodium fluoride (NaF), Genistein, Bradford reagent, Luminol, Coumaric acid and Lactate Dehydrogenase Activity Assay Kit (MAK066) were purchased from Sigma (Missouri, USA). RPMI-1640 media and other chemicals like antibiotics, amino acids etc were purchased from HIMEDIA (Mumbai, India) The fetal bovine serum (FBS) was purchased from HyClone (Thermo Scientific Hy-Clone, Logan, Utah) and Methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sisco Research Laboratory (Mumbai, India). Antibodies such as anti-Caspase-3 (ab47131), anti-Caspase-8 (ab25901) and HRP (ab97051) were purchased from Abcam (Cambridge, UK). Anti-Caspase-9 (#9508) and anti β-Actin (#4970) were purchased from Cell Signaling Technology (Danvers, MA 01923). Other essential chemicals used were of the analytical grade.

Cell culture

The normal kidney epithelial (NKE) cells were used in this study. This cell line was obtained from the uninvolved kidney tissue of a patient suffering from renal cell carcinoma, following which they were immortalized via transduction (by the human telomerase subunit) [27]. The NKE cells were maintained in RPMI medium supplemented with 10% FBS and antibiotics. They were kept at 37°C in 75 cm^2 culture flasks in a humidified incubator with 5% CO_2 and at 80% confluency the cells were subjected to the various treatments.

DPPH radical scavenging activity

Pure mangiferin used in this study was isolated and then characterized in our laboratory**.** The antioxidant potential of mangiferin and genistein, in cell-free system was evaluated by DPPH assay which determines the free radical scavenging activity of both. 2 ml of 125 µM DPPH solution was mixed with 2 ml of test samples having varied concentrations (0-200) µM of mangiferin,and genistein respectively, in separate tubes. These solutions were then incubated for 30 minutes in the dark at room temperature following which the absorbance at 517 nm was measured spectrophotometrically [28].

Superoxide radical scavenging activity

The superoxide radical-scavenging activity was measured for both mangiferin and genistein in separate test tubes (0-200) µM, following the method of Siddhuraju and Becker. A mixture of pH 7.4, containing phosphate buffer (0.1 M), nitrobluetetrazolium (150 µM), phenazine methosulfate (60 µM), NADH (468 µM), and various concentrations of mangiferin and genistein, was incubated in the dark at 25°C for 10 minutes, Then the samples (performed in triplicate) absorbance was read at 560 nm [29].

Hydroxyl radical scavenging activity

The hydroxyl radical-scavenging activity of both mangiferin and genistein (0-200) µM was determined following the method of Nash. Briefly, by spectrophotomerically (at 412 nm) computing the amount of formaldehyde produced from the oxidation of dimethyl sulfoxide (DMSO), the measurement of hydroxyl radicals was carried out [30].

Nitric oxide radical scavenging activity

For both mangiferin and genistein, the nitric oxide radical-scavenging activity was analysed following the method of Green et al. calculated as the percentage inhibition of nitric oxide. The samples (in triplicates) absorbance at 540 nm was determined spectrophotometrically [30].

Effect on cell viability

The NKE cells viability was assessed by the MTT assay following the methods of Sinha et al. 2014. At first, at a density of 5×10^4 cells/well, the cells were seeded in a 96-well culture plates. Control cells were treated with vehicle. NKE cells were exposed to different concentrations of NaF (100 µM-4 mM) diluted in serum-free media for twenty-four hours to determine the optimum dose (LC_{50}) of toxicity [31]. For assessing the effect of Mangiferin and genistein, they were dissolved in 0.1% DMSO was given to cells at varied concentrations (5-60 µM) for specified time. The time-dependent study (0-24 hours) on the exposure of LC_{50} dose of NaF was also carried out to determine the optimum time of NaF toxicity. Then, the cells were subjected to various concentrations of mangiferin (5-60 µM) and Genistein (5-60 µM), 2 hours prior tBHP exposure to assess their protective action. After completion of treatment with a specific molecule, to determine the cell viability, the media was discarded and the cells were washed (with PBS) and the MTT solution at a concentration of 5 mg/ml dissolved in serum-free media was added to each well following incubation at 37°C for 4 hours. To dissolve the precipitation, 100 µl of DMSO was added and the absorbance was detected at 570 nm spectrophotometrically.

Estimation of LDH leakage

To further confirm the effect of these molecules on renal cells, Lactate dehydrogenase (LDH) release in the medium was estimated with the help of the Lactate Dehydrogenase Activity Assay Kit (MAK066) manufactured by Sigma-Aldrich. The experiment was performed as described by Fotakis et al. and the activity of LDH in the medium was determined in form of absorbance at 450 nm, following the kits protocol [32].

Detection of the mode of cell death by flowcytometry

For assessing the mode of NKE cell death, the cells were cultured to 80% confluencyin 6-well plates. Then the cells were incubated at desired doses and time points in serum-free medium following which the cells were scraped and then centrifuged (300 × g, 5 min). The pellets of each sample thus obtained were resuspended in Annexin V Binding Buffer (1X) to which 1 µl of Annexin V/FITC was added and incubated in dark (5 minutes) at room temperature. Immediately, the samples with an excitation (488 nm) and emission (520 nm) wavelength were analysed flowcytometrically [33].

Measurement of intracellular ROS

Approximately, NKE cells ($2 \times 10^{6)}$ were exposed to optimum doses of NaF, mangiferin and genistein and incubated, scraped and then pelleted by centrifugation for 5 minutes (300 g). After that the cell pellet was suspended in 1 ml of PBS to which H_2DCFDA at a final concentration of 2 µM was added. Then the samples were incubated in the dark (20 minutes, 37ºC) followed by analyses of the samples with FACS Verse via FAC-Suite software, at 488 nm and 520 nm (the excitation and emission wavelength) respectively [34].

Assessment of lipid peroxidation

The intracellular lipid peroxidation was measured following the method of Esterbauer and Cheeseman. Briefly, following cell treatments, the cells were suspended in a buffer, sonicated, mixed with 20% of trichloro aceticacid and 0.67% thiobarbituric acid, heated for 1 hour at 100°C and cooled. After this the precipitate was removed by centrifugation and the absorbance (535 nm) was measured [8].

Analysis of ferric reducing/antioxidant potential

The Ferric reducing antioxidant power of mangiferin and genistein was determined by FRAP assay. Briefly, 50 µl of the experimental cell suspension were added respectively in separate tubes to 1.5 ml of freshly prepared pre warmed FRAP solution reagent (300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1). The mixture was then incubated for 10 min at 37°C and at 595 nm the absorbance was measured against the reagent blank [6].

Estimation of the non-enzymatic cellular metabolites

Non-enzymatic cellular metabolites or cellular GSH levels were determined following the protocol of Ghosh et al. To the supernatant collected after treatment, sonication and centrifugation, DTNB solution (Ellman's reagent) was added and the absorbance (412 nm.) was measured. The level of GSSG (oxidized GSH) was determined according to the method of Hissin and Hilf. After adding the different chemicals, the absorbance (420 nm) was recorded spectrophotometrically [8, 35].

Assesment of the intracellular antioxidant enzymes

The activity of different antioxidant enzymes viz., superoxide dismutase, catalase, glutathione S-transferase, glutathione reductase and glutathione peroxidase were determined spectrophotometrically according to the method described by Rashid et al [36].

Evaluation of the mitochondrial membrane potential

The mitochondrial membrane was determined flowcytometrically following the protocol of Salvoli et al. with help of the JC1 dye in BD FACS Calibur Flow Cytometry System (excitation: 485 nm; emission: 530 nm, 590 nm) (BD Biosciences) [37].

Cell cycle analysis

Cell cycle was analysed flowcytometrically. Briefly, following NKE cells desired treatments, the cells were washed with cold PBS and mixed with a hypotonic solution (sodium citrate, titron X,

propidium iodide), and RNase A. Then the mixture was incubated for 15 mins in dark at 4°C and the stained cells was measured by FACS [38].

Immunoblotting

The cells were lysed,sonicated and centrifuged for protein isolation following which the protein was estimated by Bradford assay.

For western blot analysis equal amount of protein were loaded in each well and resolved by SDS-PAGE, transferred to PVDF membrane, incubated with the desired primary antibody (for overnight) and secondary antibody (for 2 hours) and lastly, developed by the HRP substrate ECL solution containing Tris-HCl, Luminol, Coumaric acid and Hydrogen peroxide [39].

Statistical analysis

After independent experiments (3), results are expressed as mean data (± SD). Statistical evaluation has been undertaken by the means of ANOVA and Tukey test for comparing the group means. A p-value was considered as statistically significant if it was less than 0.05.

Results

Mangiferin and genistein possesses radical scavenging activity

Mangiferin and genistein both exhibited strong free radical scavenging activities. In **Figures 1a-1c** it was shown that mangiferin and genistein both had the potential to scavenge the DPPH, superoxide, nitric oxide and hydroxyl radicals in a cell free experimental set up. It was also observed that free radical scavenging activity of mangiferin **(Figures 1a and 1c)** was higher at lower doses compared to genistein **(Figures 1a and 1b).** However, at higher doses both the compounds showed similar activity.

NaF induces dose and time dependent toxicity on NKE cells

Results of the dose and time-dependent toxic effect of NaF were presented in (**Figure 2a, 2b and 2d).** It was observed that NaF decreased the cell viability by 50% at 500 µM dose, when exposed to 24 hours. Later, time dependent assay revealed that NaF could induce cytotoxicity in about 16 hours of time and beyond this time point the effect of NaF was not significantly altered. In all the experiments performed in this study, NKE cells were exposed to 500 µM of NaF for 16 hours.

Effect of antioxidants on NaF induced cytotoxicity

Previous data suggest that NaF causes a decrease in cell viability, dose dependently. As shown in **(Figure 2e)**. We found that both mangiferin and genistein, when pre-treated (2 hours), were effective in resisting the effect of 500 µM NaF (incubated for 16 hours). Both the antioxidants were used at a concentration of 5, 10, 20, 40 and 60 µM dose 2 hours prior to the NaF exposure in NKE cells. It was observed that mangiferin and genistein showed maximum protective effect against the oxidative stress at 5 and 10 µM respectively. From the next experiments onwards, these doses were used for the antioxidant pre-treatment. It was also observed that, in absence of the oxidative insult, mangiferin and genistein exerted no toxic effect on the NKE cells till 40 µM, after which they became slightly toxic **(Figure 2c).**

Mangiferin and genistein pretreatment prevents LDH leakage

NaF exposure to the NKE cells for 16 hours induced LDH leakage in the culture medium. From the experimental data presented in the **(Figure 3)**. it is depicted that both mangiferin and genistein effectively reduced the toxic effect of NaF, as the level of LDH leakage was significantly less in the antioxidant pre-treated cells.

Effect on cell death

To observe the effect of mangiferin and genistein upon NaF induced cell death, flow cytometric analysis was carried out to estimate the percentage of the cells undergoing either apoptosis or necrosis. Experimental data revealed **(Figure 4)** that in NaF treated group, the percentage of early apoptotic cell was around 60% higher compared to the control cells. Mangiferin and genistein treated groups did not show any significant change compared to control but when the antioxidant pre-treated cells were exposed to NaF for 16 hours, it was observed that the percentage of apototic cells were greatly reduced.

Effect on intracellular ROS generation

Figure 5 presented the level of intracellular ROS in the control and experimental NKE cells. It was observed that under normal culture condition the polyphenolic compounds did not have any effect on the ROS level. NaF exposure increases the intracellular ROS level almost two fold compared to the control cells. It was also observed that mangiferin and genistein is potential in maintaining the ROS level same as control NKE cells, when the renal cells were subjected to them before NaF exposure for 16 hours.

Effect on lipid peroxidation and FRAP

NaF exposure increased MDA level in the NaF exposed experimental cells compared to control cells. Mangiferin as well as genistein was effective in resisting the change induced by NaF due to the oxidative insult. The experimental data presented in **(Figure 6a)** also suggested that in absence of the NaF exposure, mangiferin and genistein showed no significant effect on lipid peroxidation compared to the control cells. Along with this NaF also decreased the ferric reducing/antioxidant power in the NKE cells **(Figure 6b**). In line with the previous data, it was observed that mangiferin and genistein exposure inhibited the reduction significantly compared to the only toxin treated cells.

Effect on cellular metabolites

Exposure to NaF significantly reduced the level of intracellular GSH along with the increased the GSSG level. Thus, overall it decreased the ratio of GSH and GSSG. It was also observed that mangiferin and genistein positively maintained the intracellular

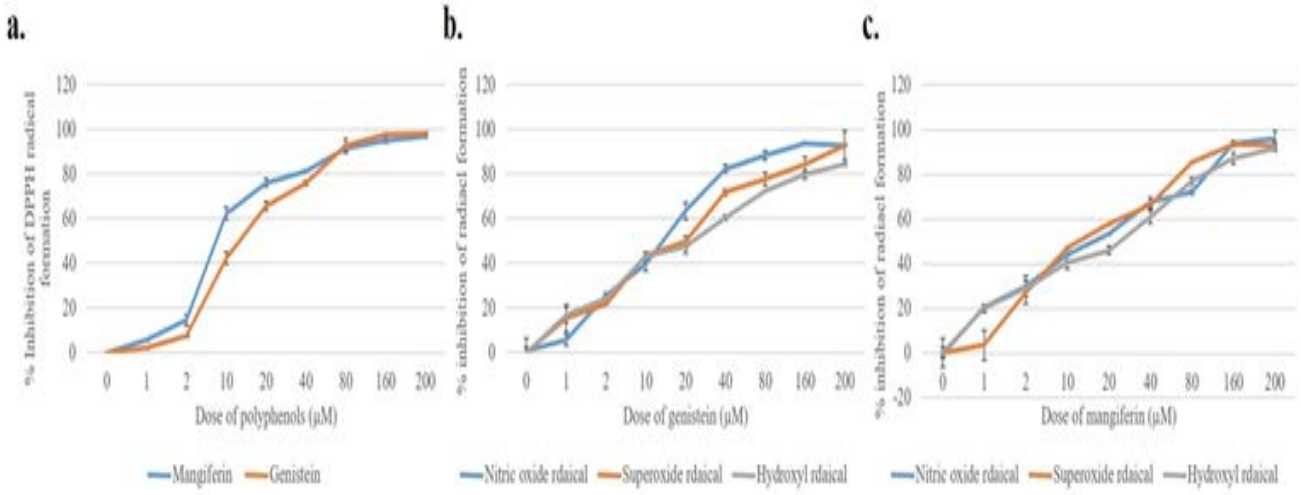

Figure 1 **a)** DPPH radical sacvenging of mangiferin (MAG) and genistein (GEN). **b**) Superoxide radical, hydroxyl radical and nitric oxide scavenging activity of genistein in the cell-free system. **C)** Superoxide radical, hydroxyl radical and nitric oxide scavenging activity of genistein in the cell-free system. Each point represents the mean ± SD, n=6.

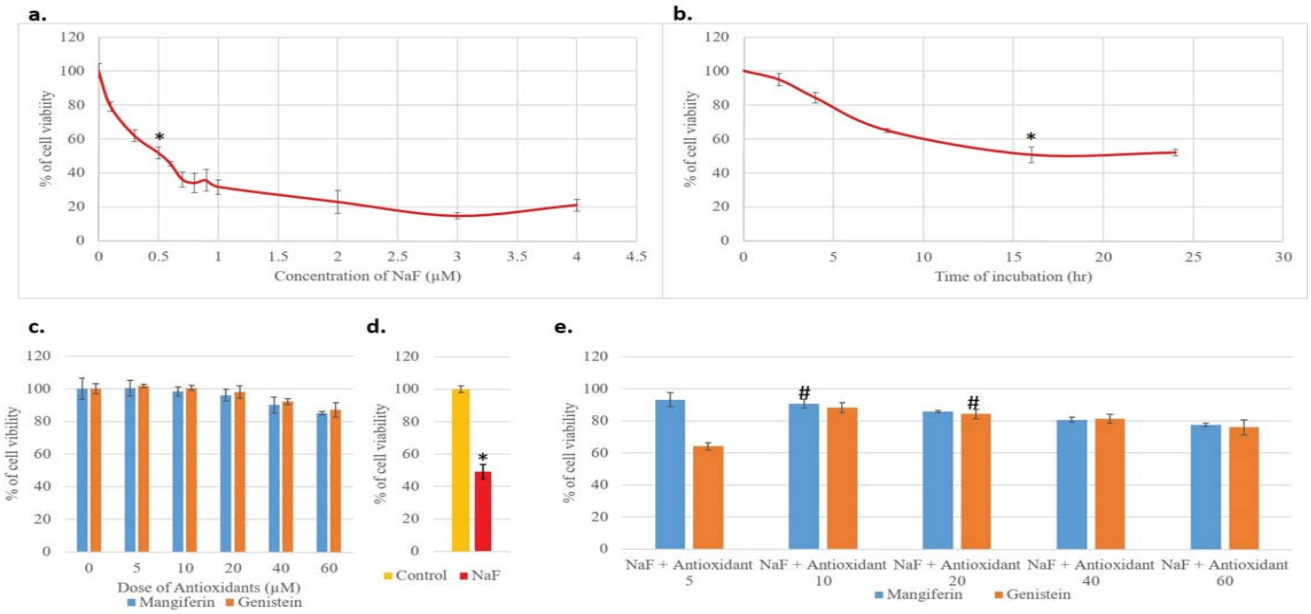

Figure 2 **a)** Dose dependent effect of NaF on NKE cells were presented, cells ere treated for 24 hr. **b)** Time dependent effect of 500 µM NaF on NKE cells. **c)** Effect of mangiferin and genistein on the cell viability of NKE cells. **d)** Effect of 500 µM NaF was comapered with the control/untreated cells. **e)** Dose dependent effect of mangiferin and genistein on 500 µM NaF exposed cells. Each point represents the mean ± SD, n=6. ''*'' represents the significant difference between the normal control and NaF exposed cells and ''#'' represents the significant difference between the NaF exposed and NaF exposed antioxidant treated cells (P*<0.05, P#<0.05).

GSH-GSSG ratio upon NaF induced oxidative insult. The data is represented in graphical format in **(Figure 7a)**.

Effect on the endogenous antioxidant enzymes

The graphical data presented in **(Figure 7b)** showed the activity of several endogenous antioxidant enzymes, such as SOD, CAT, GST, GR and GPx on exposure to toxin and protective agents. It was clearly observed the NaF exposure decreased the activity of all the enzymes used in this particular study. Pre-treatment with the antioxidants maintained the enzyme activity compared to the control cells.

Effect on mitochondrial membrane potential (MMP)

The effect of NaF induced oxidative stress on the mitochondrial membrane potential has been depicted in the **(Figure 8)**. It

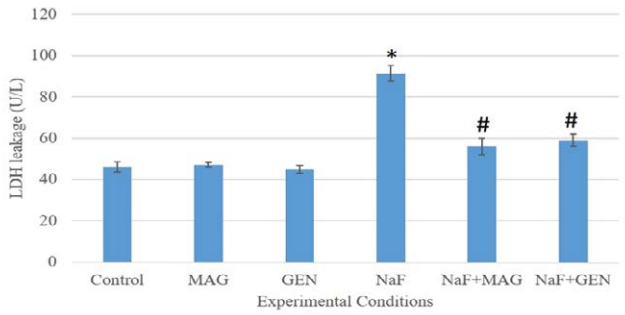

Figure 3 Effect of NaF on NKE cells as measured by LDH leakage assay [unit/litre]. Each point represents the mean ± SD, n=6. ''*'' represents the significant difference between the normal control and NaF exposed cells and ''#'' represents the significant difference between the NaF exposed and NaF exposed antioxidant treated cells (P*<0.05, P#<0.05).

was observed that 500 µM of NaF significantly reduced the mitochondrial membrane potential compared to the normal cells; whereas when the cells were pre-treated with the mangiferin and genistein prior to the oxidative insult, the mitochondrial membrane potential was maintained as that of the control cells. Mangiferin and genistein alone had no significant effect on the MMP of the renal cells.

Effect of NaF on cell cycle progeression

Flowcytometric analysis, presented in the **(Figure 9)**, depicted that NaF potentially arrested the cell cycle progression at G0/G1 phase. Pre-treatment with the both the antioxidants relieved the cellular stress and maintained the normal progression of cell cycle, when exposed to NaF.

Effect on the key regulatory proteins of apoptotic pathway

To further explore the molecular mechanism targeted by NaF, immunoblot analyses were carried out. It was observed that exposure to NaF significantly induced the expression of several apoptosis regulatory proteins by activating them. **Figure 10** shows the levels of the activated forms caspase 8, caspase 9 and caspase 3. When the renal cells were exposed to the antioxidants prior to NaF exposure, the expression levels of the above mentioned proteins was found to be decreased compared to the only NaF treated cells. For the only antioxidant exposed cells the activated level of these proteins were found to remain equal compared to the normal cells.

Discussion

Reactive oxygen species (ROS), having a wide range of pathogenic properties, are the collection of different free radicals, peroxides, inorganic and organic oxygen ions commonly generated from the biological reduction of molecular oxygen O_2 [40]. Thus both in its chronic and acute overproduction, ROS ultimately lead to a situation popularly known as oxidative stress, causing oxidative impairment to cells and tissues [41, 42]. This is usually triggered by different kind of toxin exposure and result in the development of various kinds of organ pathophysiology as the natural antioxidant defence capacity of the body gets perturbed causing damage to macromolecules (DNA, proteins and lipids) [43, 44]. The antioxidant machineries of the body include intracellular antioxidant enzymes, the first line of cellular defense, that prevents biological macromolecules from oxidative stress and glutathione antioxidant systems, the second line of cellular defence that play a pivotal role in neutralizing free radicals and different oxidant species.

In the present study, two polyphenolic isoflavonoids, mangiferin and genistein, showed significant free radical scavenging activity, confirmed by different assays in cell-free experimental systems. Apart from the free radical scavenging activity, these two molecules proved to be potential in modulating the expression and activity of different intracellular signalling molecules [16, 22]. This study demonstrated the protective role of these antioxidant molecules in normal kidney epithelial cells against exogenous oxidative insult by sodium fluoride. The extent and nature of toxicity of a toxin varies greatly upon the cell types used in the study, e.g. sodium fluoride induces optimum level of toxicity in spermatozoa at a concentration of about 250 mM. Again when hepatocytes were treated by NaF, it was reported that 100 mM of NaF is potential enough to induce significant necrotic cell death [6]. Many more scientific reports suggest that the optimum toxic dose (LC_{50}) of a specific toxin depends solely upon the sensitivity of the particular cell type against a certain toxin. In our study we observed that the LC50 dose of NaF on NKE cell is 300 µM. In all the experiments performed in this study, NKE cells were intoxicated with 300 µM of NaF. The sensitivity of the cells towards toxin is also applicable for polyphenols too. We used both the compounds at varied doses and found that mangiferin and genistein exert optimum protection at 5 µM and 10 µM respectively. For both the isoflavones, it was observed that exposure to the antioxidants prevented the induction of cellular toxicity due to NaF, and the cell viability of the protective treated groups was nearly same as the control cells. It is well established that the first indication of cellular stress is disintegration of the cell membrane, i.e., the leakage of the cytosolic enzymes in the culture media [45, 46]. We measured the level of such a cytosolic enzyme, LDH, in the cytosol and found that NaF-intoxicated NKE induces LDH leakage. However, treatment with the antioxidants maintains the integrity of the plasma membrane upon NaF exposure. After confirming the cytotoxicity induced by NaF and getting an indication that both of the polyphenolic compounds is potentially resisting the toxic effect of NaF, FACS analysis was done with two fluorescent dye Annexin V and PI. This experiment leads us to conclude that exposure to 300 µM NaF for 16 hrs induces apoptotic cell death in the renal cells. We again found that NaF exposure causes an elevation of intracellular ROS in the cells, which was found to be effectively inhibited upon the pre-exposure of the antioxidants. Since ROS level measurement is not the direct measurement of the impact and preventive role on oxidative stress, the level of lipid peroxidation was evaluated and we found that MDA level got significantly increased in NaF treated cells and this increment in the level of MDA was resisted by the pre-treatment of mangiferin and genistein.

From the previous experiments we got a definite picture that NaF induces oxidative stress mediated apoptosis in the renal cells and both these antioxidant molecules, mangiferin and genistein potentially resisted the effect of exogenous oxidative insult. We performed several assay to quantify some antioxidative cellular metabolites and enzymes which always maintain an intracellular homeostasis between the free radicals and antioxidant levels. It was observed that NaF significantly impaires the endogenous antioxidant machinery and pre exposure to the polyphenols facilates the cell to resist the effect of NaF.

In line with the previous experiments we have also monitored the mitochondrial health, and found that in NaF exposed cells mitochondrial membrane potential is significantly depleted,

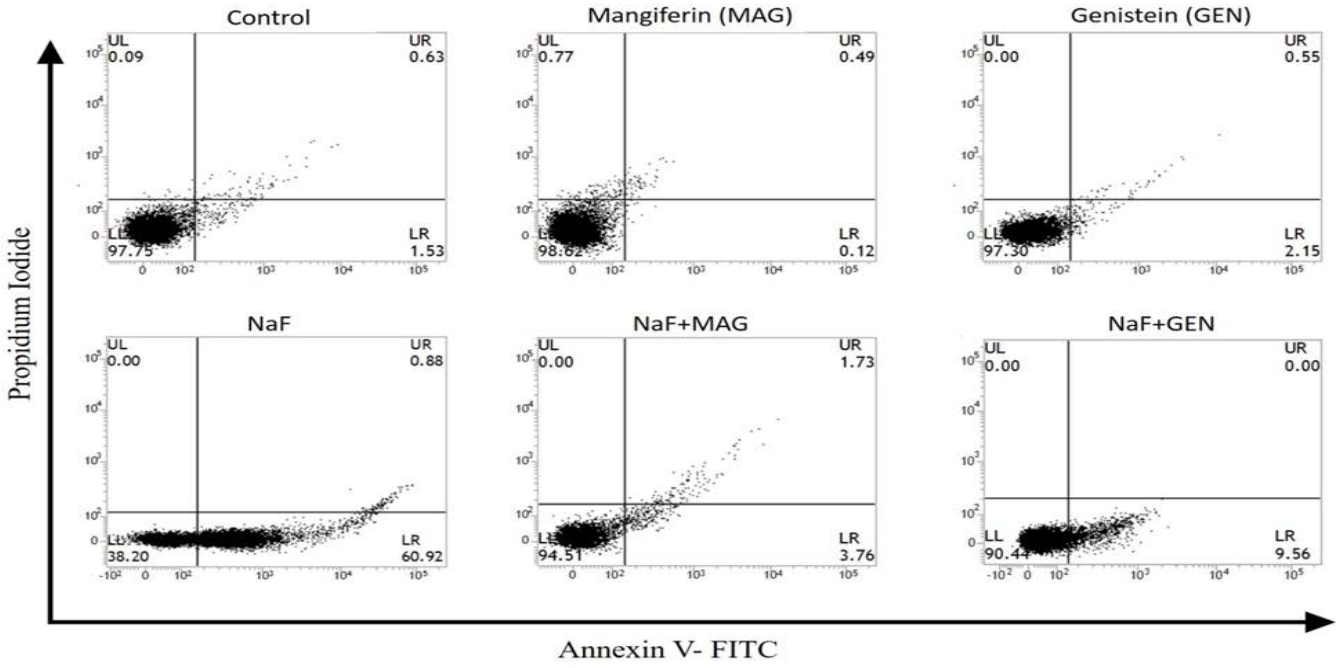

Figure 4 Detection of NaF induced mode of cell death, anti-apoptotic effect of mangiferin and genistein on NKE cells via Annexin V-FITC staining. The NKE cells were incubated separately with desired molecules (NaF, Mangiferin and Genistein) Dual parameter dot plot of FITC-labelled Annexin V fluorescence (x-axis) versus PI-fluorescence (y-axis) has been shown in logarithmic fluorescence intensity. Quadrants: lower left, live cells; lower right, apoptotic cells; upper left, necrotic cells. Data are representative of three independent experiments.

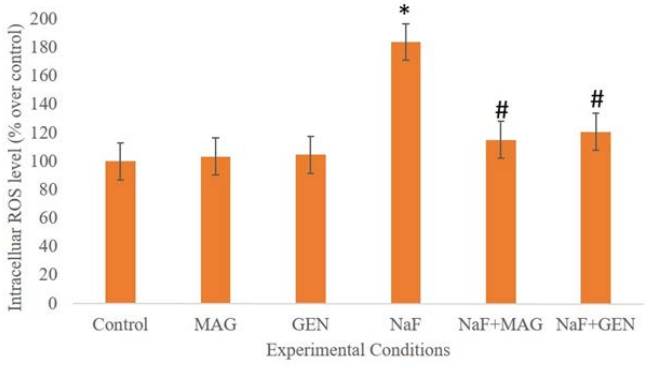

Figure 5 Effect of NaF on NKE cells as measured by intracellular ROS level. Each point represents the mean ± SD, n=6. ''*'' represents the significant difference between the normal control and NaF exposed cells and ''#'' represents the significant difference between the NaF exposed and NaF exposed antioxidant treated cells (P*<0.05, P#<0.05).

but pre-treatment with mangiferin and genistein exclusively, have effectively resisted the change in membrane potential and exhibited the potential close to that of control cells. It was also observed that NaF significantly arrested the cell cycle at G0/G1 stage, cell percentage in S and G2 phase got significantly increased. This change in the cell cycle was also effectively resisted by the antioxidants pre-treatment.

Confirming that NaF induces apotosis in the renal cells which gets resisted by the mangiferin and genistein pre-treatment exclusively, we checked the expression level of some key regulatory proteins in the apoptotic pathway and inferred that NaF significantly induces both the intrinsic and extrinsic pathway of apoptosis, as it increases the expression of caspase 8, caspase 9 and caspase 3 [9]. In antioxidant pre-treated cells we found the expression level of these apoptotic regulatory proteins are at par with the control cells. The overall observations of this study has been schematically presented in **Figure 11**, where in summary, it shows that NaF exposure in normal kidney epithelial cells induces cytotoxicity, disintegrates the plasma membrane, induces apoptosis, elevates intracellular ROS, impairs the endogenous antioxidant machinery, arrests cell cycle progression and modulates apoptosis regulatory proteins. Pre-treatment with both mangiferin and genistein exerts no toxicity in the renal cells, rather maintains the normal cellular homeostasis against NaF induced oxidative damage. Although this study represents the cyto-protective activity of both these isoflavonoids, further studies are, however, required to reveal their detailed molecular mechanism both *in vitro* as well as *in vivo* models.

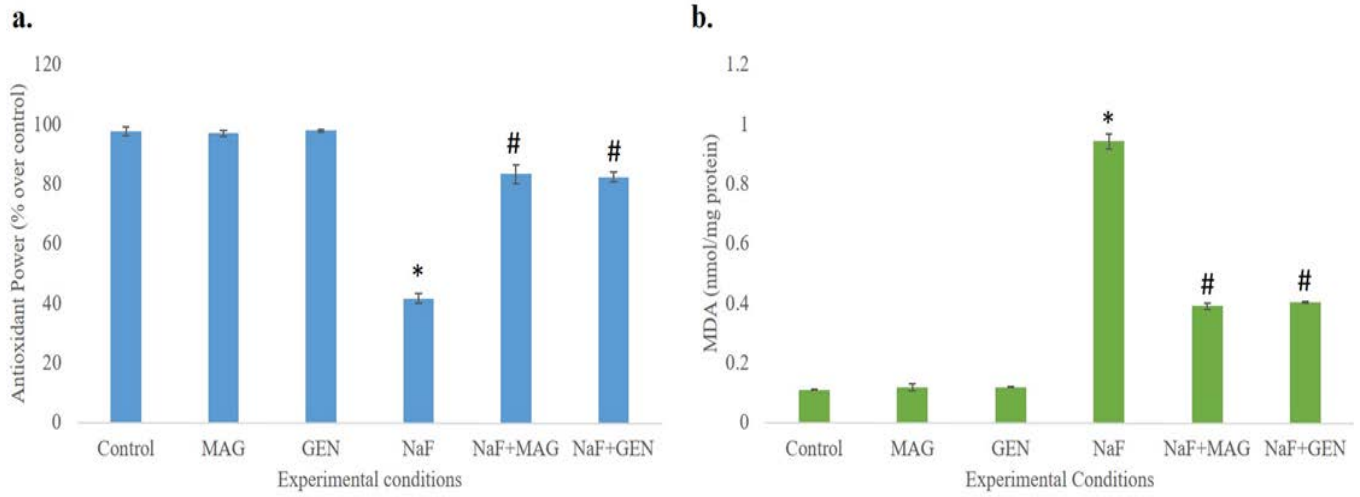

Figure 6 Effect of mangiferin (MAG) and genistein (GEN) on a) intracellular antioxidant activity (FRAP) and b) Lipid peroxidation in renal cells upon NaF exposure. Each point represents the mean ± SD, n=6. ''*'' represents the significant difference between the normal control and NaF exposed cells and ''#'' represents the significant difference between the NaF exposed and NaF exposed antioxidant treated cells (P*<0.05, P#<0.05).

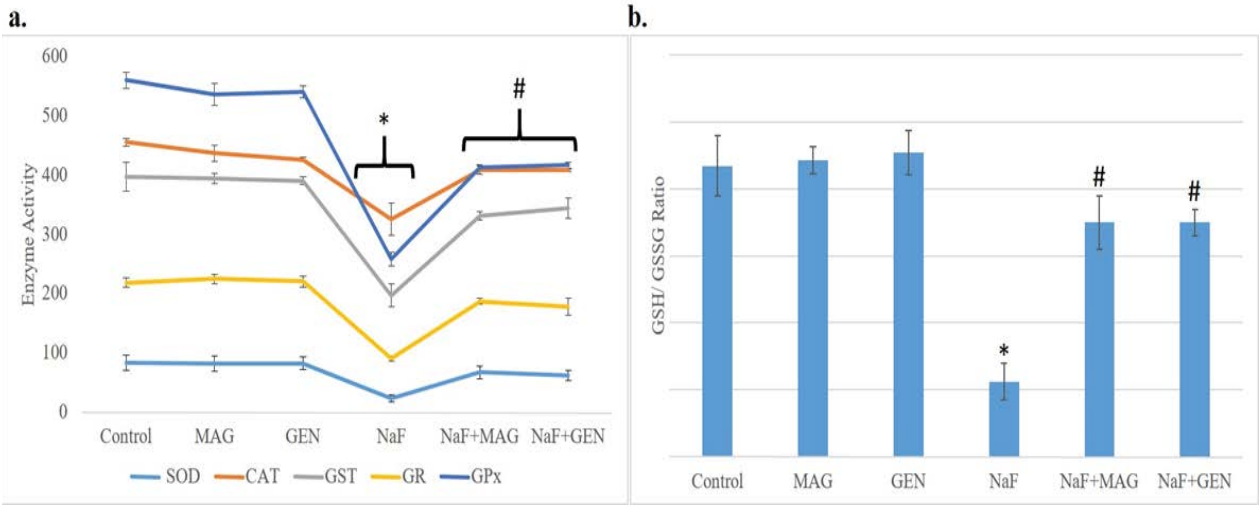

Figure 7 (a) Effect of NaF, mangiferin and genistein on the activities of phase II antioxidant enzymes in NKE cells. (b) Effect of NaF, mangiferin and genistein on GSH to GSSG ratio, Each column represents mean SD, n=6. ''*'' represents the significant difference between the normal control and NaF exposed cells and ''#'' represents the significant difference between the NaF exposed and NaF exposed antioxidant treated cells (P*<0.05, P#<0.05).

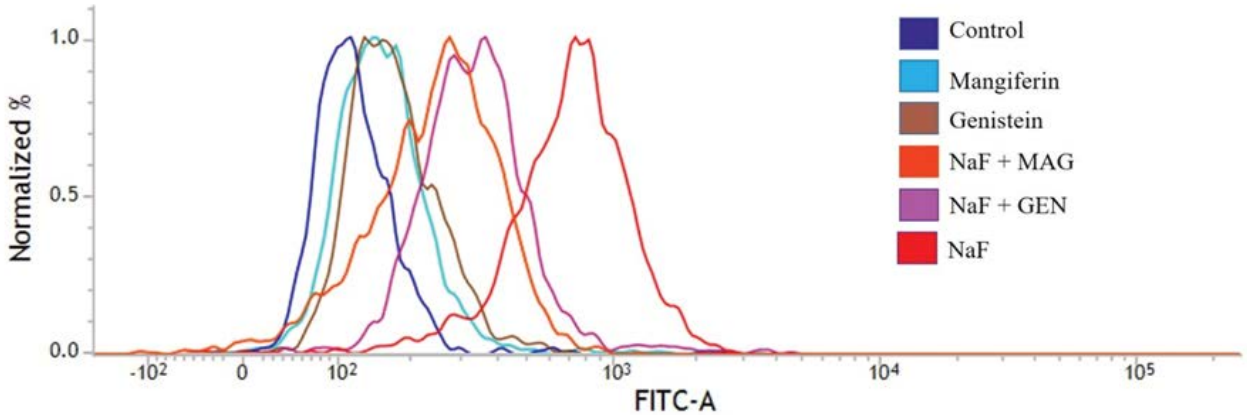

Figure 8 Effect of NaF, mangiferin and genistein on the mitochondrial membrane potential in NKE cells as shown by the monomeric green fluorescence of the JC 1 dye. Data are representative of three independent experiments.

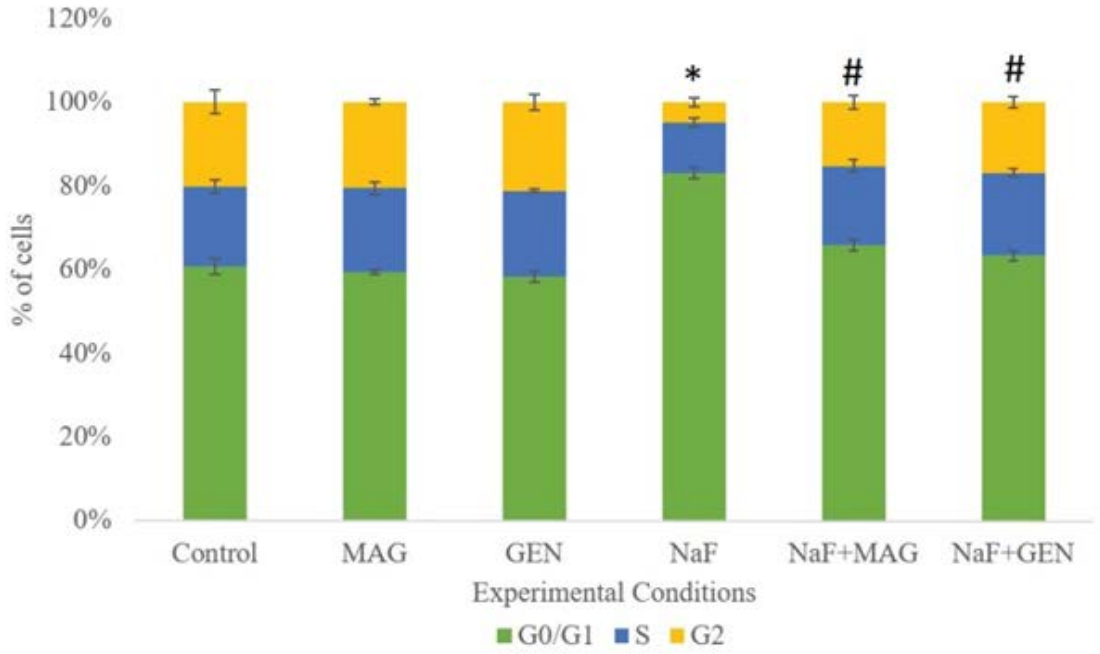

Figure 9 Cell cycle analysis shows that NKE cells when exposed to NaF showed cell cycle arrest on G0/G1 phase. Pretreatment with mangiferin and genistein inhibited the toxic effect of NaF. ''*'' represents the significant difference between the normal control and NaF exposed cells and ''#'' represents the significant difference between the NaF exposed and NaF exposed antioxidant treated cells (P*<0.05, P#<0.05).

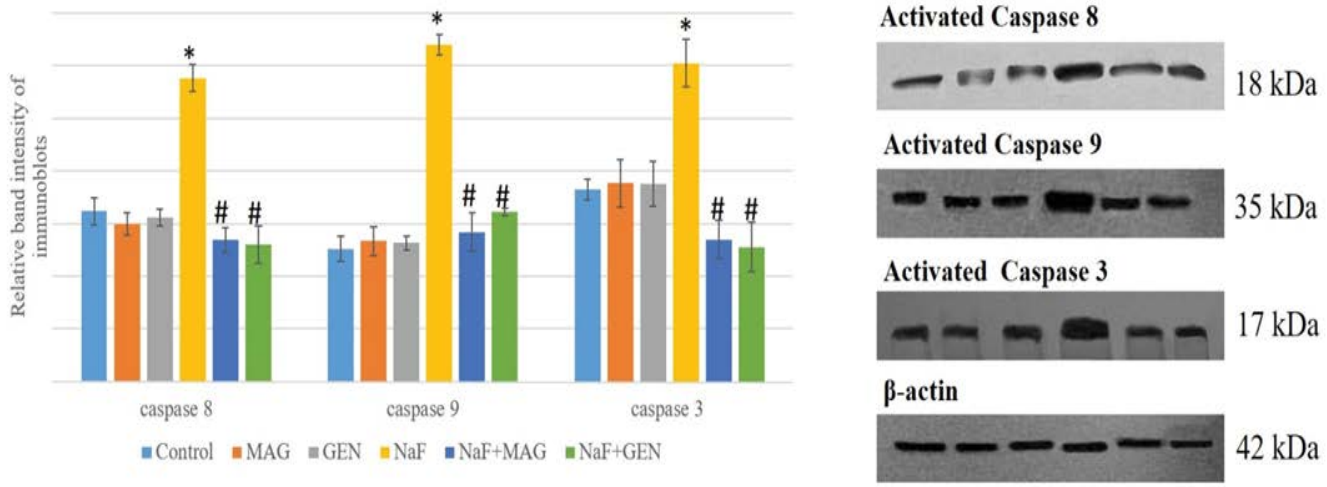

Figure 10 Immunoblot analysis of activated (cleaved) Caspase 8, Caspase 9 and Caspase 3 in the experimental in NKE cells. All data are mean ± SD, for 3 independent experiments and were analyzed by one-way ANOVA. ''*'' represents the significant difference between the normal control and NaF exposed cells and ''#'' represents the significant difference between the NaF exposed and NaF exposed antioxidant treated cells (P*<0.05, P#<0.05).

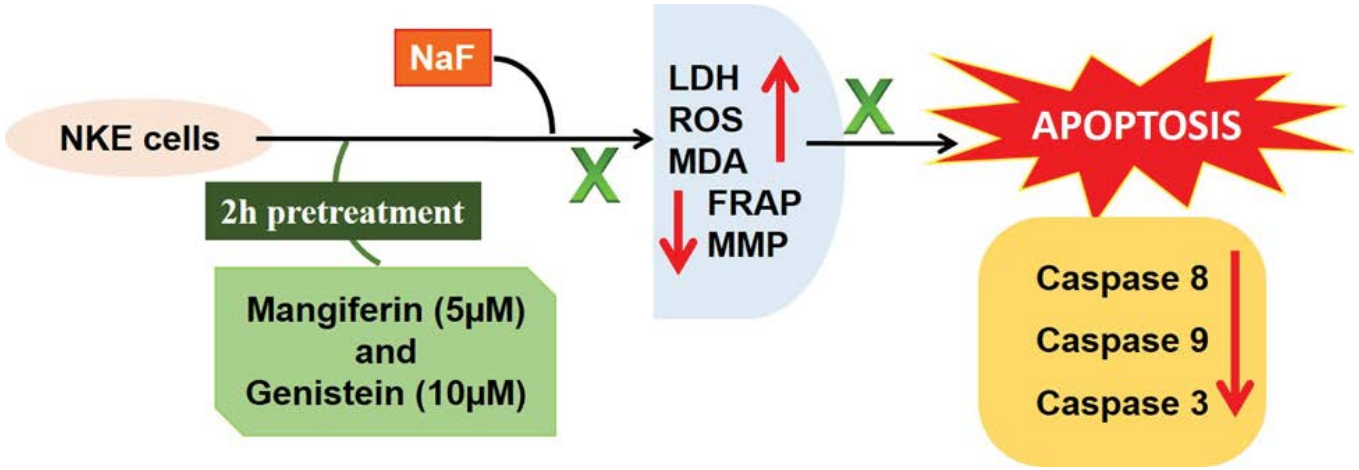

Figure 11 Graphical representation of the overall study. The red marks indicate the effect of NaF and the green represent the effect of antioxidant molecules.

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