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Mangiferin attenuates oxidative stress induced renal cell damage through activation of PI3K induced Akt and Nrf-2 mediated signaling pathways

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ABSTRACT

Background: Mangiferin is a polyphenolic xanthonoid with remarkable antioxidant activity. Oxidative stress plays the key role in tert-butyl hydroperoxide (tBHP) induced renal cell damage. In this scenario, we consider mangiferin, as a safe agent in tBHP induced renal cell death and rationalize its action systematically, in normal human kidney epithelial cells (NKE).

Methods: NKE cells were exposed to 20 μM mangiferin for 2 h followed by 50 μM tBHP for 18 h. The effect on endogenous ROS production, antioxidant status (antioxidant enzymes and thiols), mitochondrial membrane potential, apoptotic signaling molecules, PI3K mediated signaling cascades and cell cycle progression were examined using various biochemical assays, FACS and immunoblot analyses.

Results: tBHP exposure damaged the NKE cells and decreased its viability. It also elevated the intracellular ROS and other oxidative stress-related biomarkers within the cells. However, mangiferin dose dependently, exhibited significant protection against this oxidative cellular damage. Mangiferin inhibited tBHP induced activation of different pro-apoptotic signals and thus protected the renal cells against mitochondrial permeabilization. Further, mangiferin enhanced the expression of cell proliferative signaling cascade molecules, Cyclin d1, NFκB and antioxidant molecules HO-1, SOD2, by PI3K/Akt dependent pathway. However, the inhibitor of PI3K abolished mangiferin's protective activity.

Conclusions: Results show Mangiferin maintains the intracellular anti-oxidant status, induces the expression of PI3K and its downstream molecules and shields NKE cells against the tBHP induced cytotoxicity.

General significance: Mangiferin can be indicated as a therapeutic agent in oxidative stress-mediated renal toxicity. This protective action of mangiferin primarily attributes to its potent antioxidant and antiapoptotic nature.

1. Introduction

Nephropathy or impairment of kidney function is closely associated with renal toxicity. This toxicity may occur due to various endogenous and exogenous toxins (like environmental pollutants, drugs, metals, etc.) leading to physiological dysfunctions accompanying a wide variety of symptoms [1]. Scientific evidence suggests oxidative insult to be one of the primary phenomena leading to renal dysfunction [2,3], a very eminent problem of the modern day world. So, making a nephrotoxic *in vitro* model by intoxicating renal cells with an oxidative stress causing agent is rational. An organic peroxide, tert-butyl hydroperoxide (t-BHP) is widely used as a classic inducer of oxidative stress in many studies [4,5]. tBHP is a major environmental pollutant causing increased ROS (Reactive Oxygen Species) formation in cells and is metabolized by two independent pathways either by the formation of end products like peroxyl and alkoxyl radicals [6] or oxidized glutathione (GSSG) [7,8]. Thus, whichever may be the pathway of its metabolism, the ultimate outcome is the induction of oxidative injury. This molecule even leads to programmed cell death or apoptosis in different cells and tissues [9–11]. In addition, tBHP is also known to induce renal lesions, thus acting as a nephropathic agent also [12,13]. So, the administration of this exogenous oxidative stress inducer may simulate a situation of augmented oxidative insult and damage in normal kidney epithelial cells and helps us to understand particular mechanisms in the pathogenesis of oxidative stress induced nephropathy.

Interestingly, mammalian cells posess defense mechanisms (both enzymatic and non-enzymatic) to prevent ROS formation or

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to detoxify the already produced ROS [14]. However, these multifunctional defensive systems cannot totally counteract the deadly effects of amplified ROS, an outcome observed in different pathophysiological states [15], i.e. the physiologically available amount of antioxidant enzymes is not sufficient to counteract pro-oxidants in most cases. Thus targeting the imbalance between antioxidants and pro-oxidants in cells, i.e. oxidative stress, seems to be a logical approach for treating such disorders with possibly fruitful outcome. Therefore, external supplements, having antioxidant property that can increase the level and activities of endogenous antioxidants [16–18] can be proposed as therapeutic agents to combat ROS overproduction [19,20].

Herbal antioxidants have gained special attention in the contemporary scientific community because of the acuity about their lower toxicities in comparison to synthetic molecules [21–27]. Though quite a number of pharmaceutical molecules have originated from the phytoenvironment or been derived from tissues of plants, still they represent a comparatively unexploited source of potentially novel compounds. Polyphenols are the predominant group of natural antioxidants showing powerful antioxidant activity due to their ability of inducing gene expressions of antioxidant enzymes, and having properties like free radical scavenging, hydrogen donating, singlet oxygen quenching and so on [28–31]. In particular, mangiferin (2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), derived commonly from the bark and leaves of plants belonging to Anacardiaceae and Gentianaceae families, may provide a unique and underutilized source of potential therapeutic agent [32]. This naturally occurring polyphenol is mainly isolated from the widely distributed mango tree (*Mangifera indica*) and has been in use long back even before its characterization was done, in different corners of the world, to treat disorders like melancholia, bleeding dysentery etc [33,34]. Gradually different studies revealed mangiferin's antioxidant [35,36], antitumor [32,37], antidiabetic [38,39] and immunomodulatory activities [40]. This xanthone possess the ability to scavenge ROS, a causal inducer of oxidative stress, inhibit lipid peroxidation, and increase the reduced glutathione content, thus establishing itself as a good antioxidant. Moreover, it can modulate the expression of a number of apoptosis-related genes playing very important role in regulating apoptosis.

The above-mentioned therapeutic claims made about mangiferin and some previous studies of our laboratory [38,39,41] in accordance with these claims encouraged us to evaluate its antioxidant potential in oxidative stress related nephropathy. So far, there is no study describing the protective role of mangiferin in tBHP -induced, oxidative stress mediated nephrotoxicity in normal kidney epithelial cells (NKE). In the present study, we investigate whether this polyphenol was effective in attenuating the nephrotoxicity and its mechanism of protective action, promoting better understanding of the antioxidant as well as nephroprotective properties of mangiferin. We also show that the generation of oxygen radicals by tBHP was successfully neutralized by mangiferin thereby protecting the kidney cells via PI3K/Akt pathway from oxidative stress mediated pathophysiology.

2. Materials and methods

2.1. Materials and reagents

RPMI-1640 media and other necessary chemicals like antibiotics, amino acids etc were purchased from HIMEDIA (Mumbai, India) and fetal bovine serum (FBS) from HyClone (Thermo Scientific Hy-Clone, Logan, Utah), respectively. tBHP was purchased from Sigma-Aldrich and Methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sisco Research Laboratory (Mumbai, India). Fluorescein isothiocyanate (FITC) conjugated Annexin V, Apoptosis detection kit, RNaseA, Bradford reagent, Luminol, Coumaric acid and LY294002 (a PI3K inhibitor) were also purchased from Sigma (Missouri, USA). Antibodies such as anti Caspase-3 (ab47131), anti Caspase-8 (ab25901), anti Bid (ab77815), anti Bcl2 (ab7973), anti cytochrome c (ab76237), anti Nrf2 (ab31163), anti HO1 (ab13243), anti SOD2 (ab13533), Phospho JNK (ab4821), anti Bax (ab32503), anti PI3k (ab74136), anti Akt (ab17785), Phospho Akt (ab23509), HRP (ab97051) were purchased from Abcam (Cambridge, UK). Phospho BAD (#9291), anti mTOR (#2983), anti GSK 3β (5338), anti Cyclin D1 (#2978), anti PARP (#9532), anti Caspase-9 (#9508), anti Apaf-1 (#8723), anti Bax (#2772), Anti NFκB (#3034), anti β-Actin (#4970), anti Lamin B1 (#12586) was purchased from Cell Signaling Technology (Danvers, MA 01923). Other essential chemicals used in this study were of the analytical grade.

2.2. Free radical scavenging activity of mangiferin

Mangiferin used in the present study was isolated, purified and characterized in our laboratory [38]. To evaluate the free radical scavenging potential of mangiferin and compare it to the popular antioxidant Vitamin C (Vit C), DPPH radical scavenging activity was performed in the cell-free system. The assay was performed according to the method of Blois [42]. Two ml of DPPH solution (125 μM) in methanol and 2 ml of tested samples with different concentrations (10-160) μM of mangiferin and Vit C were mixed in the tubes. The solution was incubated at room temperature for 30 min in the dark. Then the absorbance was measured at 517 nm against methanol blank using a spectrophotometer. Vit C was used as a positive control for this experiment.

2.3. 2.3. Assay of ferric reducing property

FRAP (Ferric reducing antioxidant power) assay was done according to the protocol described by Benzie et al. [43]. Briefly FRAP solution was prepared in dark at 37 °C by mixing 300 mM acetate buffer of pH = 3.6, 10 mM TPTZ in 10 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in a volumetric ratio of 10:1:1. Mangiferin was added to the final concentration ranging (10–200 μM) with 1 ml of FRAP solution warmed to 37 °C. The final solution was mixed well and incubated for 30 min at 37 °C. The Absorbance was measured spectrophotometrically at 595 nm. For positive control, we used Vit C in this experiment.

2.4. Cell culture

The normal kidney epithelial (NKE) cell line was derived from the uninvolved kidney tissue of a patient with renal cell carcinoma. The cells were immortalized by human telomerase subunit via transduction [44,45]. NKE cells were maintained at 37 °C in RPMI medium supplemented with 10% FBS and antibiotics in in75-cm^2 culture flasks in a humidified incubator enriched with 5% CO_2. Confluent monolayers (80%) of NKE were subjected to different treatments used in the studies undertaken.

2.5. Assessment of cell viability upon mangiferin treatment and tBHP exposure

Cell viability of NKE cells was determined by the MTT assay following the methods of Sinha et al. [46,47]. The cells were seeded at a density of 5×10^4 cells/well in a 96-well culture plates with 100 μl media/well. Mangiferin was dissolved in 0.1% DMSO and applied to cells at desired concentrations. Normal cells were treated with vehicle control. After the treatment with the desired compound for specified time, the media was discarded, and the

cells were washed gently twice with phosphate buffered saline (PBS). Then 5 μl of MTT solution (5 mg/ml in serum-free media) along with 45 μl serum-free media was added to each well and incubated at 37 °C. After 4 h, 100 μl DMSO was added to each well. To dissolve the precipitation completely, the plate was gently shaken for 10 min. At last, the absorbance was detected in a spectrophotometer at 570 nm.

2.6. Dose and time-dependent effect of tBHP and mangiferin

Firstly, for dose dependent study, 80% confluent NKE cells were exposed to different concentrations of tBHP (10–100 μM) diluted in serum-free media for twenty-four hours. Simultaneously, another similar study was also done in which the cells were treated for 24 h with different doses of mangiferin (5–50 μM) to check if it had any effect on the cells. The time-dependent study ranging from 0–24 h at an interval of 6 h on the exposure of LC_{50} dose of tBHP was also carried out. Thus, the optimum time of tBHP toxicity was determined by the help of this time-dependent study. Next the cells were subjected to various concentrations of mangiferin (5–30 μM) treatment for 2 h before tBHP exposure to verify its protective action. The cell viability was determined for all the groups and subgroups after completion of treatment for stipulated time with a specific molecule (mangiferin or tBHP or both).

2.7. Assessment of LDH leakage

To further confirm the renal cell damage, Lactate dehydrogenase (LDH) release in the medium was determined 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. (2006). Briefly, following exposure to the tBHP and mangiferin, at their desired doses, the culture medium was first aspirated followed by its centrifugation for obtaining cell-free media. Then the aliquots of media were mixed well with the reagent, and the absorbance was recorded spectrophotometrically at 450 nm.The activity of LDH in the medium was determined according to the kits protocol.

2.8. Determination of intracellular ROS production

This assay was performed adopting the protocol of Cossarizza [48]. Approximately, 2×10^6 NKE cells were exposed to tBHP and mangiferin at the specified dose and time. After incubation, the cells were first scraped and then pelleted by centrifugation (300 g, 5 min, RT). Then they were suspended in 1 ml of PBS and H_2DCFDA was added at a final concentration of 2 μM followed by incubation in the dark for 20 min at 37 °C. Next the analyses of the samples were carried out with FACS Verse at 488 nm and 520 nm (the excitation and emission wavelength) respectively. The results were analysed using the FAC-Suite software.

2.9. Determination Of lipid peroxidation

After desired tBHP and mangiferin exposure and following PBS wash, the cells were suspended in a buffer, pH 7.4 containing HEPES (10 mM), NaCl (130 mM), NaH_2PO_4 (1 mM), KCl (3 mM) and glucose (10 mM), and lysed by sonication. Then following the methods of Esterbauer and Cheeseman, these samples were subjected to the measurement of intracellular lipid peroxidation in the form of the end product malondialdehyde (MDA). Briefly, individual samples were mixed with 20% of trichloro acetic acid (1 mL) and 0.67% thiobarbituric acid (2 mL). The mixture was then heated at 100 °C for 1 h and then cooled for precipitation. Following this by centrifugation, the precipitate was removed from the sample and the absorbance was measured at 535 nm.

2.10. Assessment of cellular non-enzymatic antioxidant GSH

GSH is a key regulatory intracellular molecule. It potentially scavenges the ROS and its intermediates. Cellular GSH levels were determined using Ellman's reagent according to the protocol of Ghosh et al. [49]. NKE cells were harvested after the exposure protocol as described earlier, and total protein was isolated by sonication followed a centrifugation at 12000 rpm, 4 °C for 15 min. After centrifugation, the supernatant was collected, and DTNB solution (Ellman's reagent) was added to it. The absorbance was spectrophotometrically measured at 412 nm. The level of oxidized GSH, i.e., the level of GSSG was measured by following the method of Hissin and Hilf [50]. About 50 μl of the sample was mixed with 20 μl of 0.04 M NEM to prevent the oxidation of GSH to GSSG. It was incubated at room temperature for 30 min, and 1.68 ml of 300 mM Na_2HPO_4 and 250 μl of DTNB reagent were added to it. The absorbance was recorded spectrophotometrically at 420 nm.

2.11. Measurement of antioxidant enzymes

The activity of intracellular antioxidant enzymes viz., superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx) were measured spectrophotometrically according to the protocol described by Rashid et al. [51].

2.12. Analysis of cellular morphology of NKE by phase contrast microscopy

For imaging, the NKE cells were cultured in 6-well cell culture plates (BD Falcon). Then they were exposed to mangiferin and tBHP according to the specified protocol. After treatment, the medium was discarded, and the cells were washed gently with PBS. After that the cells were substituted with fresh medium and observed under phase contrast microscope (Leica Microsystem DN1000; camera: DFC450 C).

2.13. Assessment of cell death by Annexin V affinity assay

NKE cells were cultured with RPMI media in 6-well plates. At 80% confluence, the medium was substituted with serum-free medium and the cells were exposed to desired compounds in separate wells at specific doses at particular time points. After incubation, cells were gently scraped and centrifuged at 300*g* for 5 min at room temperature. The pellets were then washed with PBS and resuspended in 1 × Annexin V Binding Buffer. Then 1 μl of Annexin V/FITC was added to each sample and incubated in dark at room temperature for 5 min. The samples then were immediately analysed at FACSVerse using the FACSuite software with an excitation and emission of at 488 nm and 520 nm respectively.

2.14. DNA fragmentation assay by gel electrophoresis

Genomic DNA from NKE cells (control and exposed to 20 μM mangiferin and 50 μM tBHP either alone or both) was extracted following the method of Sellins and Cohen [52]. The extent of DNA fragmentation was assayed using 1% agarose gel electrophoresis by running extracted DNA samples in it.

2.15. Evaluation of mitochondrial membrane potential

After subjecting to required treatments, NKE cells were detached and incubated at 37 °C for 30 min with 5mM JC-1 dye. Next, for 5 min they were centrifuged at approximately 300*g* and suspended in PBS. Among them, the fluorescence-labeled ones were analyzed by a BD FACS Calibur Flow Cytometry System (excitation:

485 nm; emission: 530 nm, 590 nm) (BD Biosciences). The mitochondrial membrane potential was quantified by the ratio of fluorescence emission according to the instructions from Invitrogen (briefly following the method of Salvoli et al. [53]).

2.16. Cell lysis and immunoblotting

Cells were primarily freed from medium and lysed by using lysis buffer RIPA having a pH of 8.0 and containing 150 mM sodium chloride, Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris and protease and phosphatase inhibitors to form total cell lysates. Briefly, the cells were then vortexed, rapidly freeze-thawed thrice, sonicated and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was collected, and protein present in it was estimated using Bradford reagent. The protein samples were then aliquoted and stored at −80 °C for further use.

Next the western blot analysis was carried out in which the same amount of protein (50 μg) from each sample was loaded and resolved by 12% SDS-PAGE. After that they were transferred to PVDF membrane, the membranes were blocked to prevent non-specific binding in 5% BSA containing buffer for two hours at room temperature. Then they were incubated overnight with the desired primary antibody at its respective dilution at 4 °C. Next, for 30 min the membranes were washed in wash buffer TBST (50 mM/L Tris–HCl, pH 7.6, 150 mM/L NaCl, 0.1%Tween 20) and incubated for 2 h at room temperature with appropriate HRP-conjugated secondary antibody (1:15,000 dilution). Lastly, they were developed by the HRP substrate ECL solution containing 1 M Tris–HCl pH 8.5, 250 mM Luminol, 90 mM Coumaric acid and 30% Hydrogen peroxide [54].

2.17. Cell cycle analysis by flow cytometer

Flow cytometrical analyses were used to determine the alterations in cell cycle. NKE cells were synchronized by plating in medium containing 10% FBS for 24 h followed by culturing in RPMI medium. The cells were then supplemented into medium containing 20 μM mangiferin, 50 μM tBHP or both and harvested for 18 h after treatment, washed with cold PBS twice, and mixed with hypotonic solution containing sodium citrate, titron X, propidium iodide, and RNase A incubated in dark for 15 min in 4 °C. The fluorescence of stained cells was measured using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

2.18. Statistical analysis

After performing three independent experiments, results have been expressed as their mean data (± SD). Its Statistical evaluation has been done by the means of one-way analysis of variance (ANOVA), and Tukey test was undertaken to compare the group means. A *p*-value less than 0.05 was considered as statistically significant.

3. Results

3.1. Effect on DPPH radical scavenging and FRAP activity

The DPPH radical scavenging activities of mangiferin and Vit C in cell-free systems showed that the maximum DPPH radical scavenging activity for both the antioxidants occured at a concentration of 100 μM (Fig. 1A). Fig. 1B shows the ferric reducing property as represented the FRAP activity for both the antioxidants. In both the cases the antioxidant activity of mangiferin is found to be higher than the positive control Vit C at lower doses but similar to the former observation with DPPH it is found that at higher doses around 100 μM both the compounds exhibit maximum activity.

3.2. tBHP treated renal cells undergoes injury in a dose and time dependent manner

MTT assay and LDH release assay in tBHP-treated NKE cells were used to evaluate cell damage. tBHP led to significant cell injury in kidney epithelial cells in a dose and time-dependent manner. The dose of 50 μM for 18 h were found to be optimal as tBHP caused approximately 50% cell demise compared with the control group, in NKE cells at this specific dose and time (Fig. 2A and B).This same dose was also further confirmed by performing the LDH cytotoxicity assay; it was observed that at 50 μM of dose LDH leakage value is found to be 65 U/L (Fig. 2C). Hence, this 50 μM dose was used as the optimal toxic dose for further experimentations.

3.3. Effect of mangiferin on normal and tBHP exposed cells

MTT cell viability data suggest that at lower doses, mangiferin alone has no significant effect on NKE cells but above 30 μM dose mangiferin decreases the cell viability to some extent (Fig. 3A). Experimental data in Figs. 2A and 2B confirms that 50 μM of tBHP is potentially toxic and it decreases the cell viability around 50% in

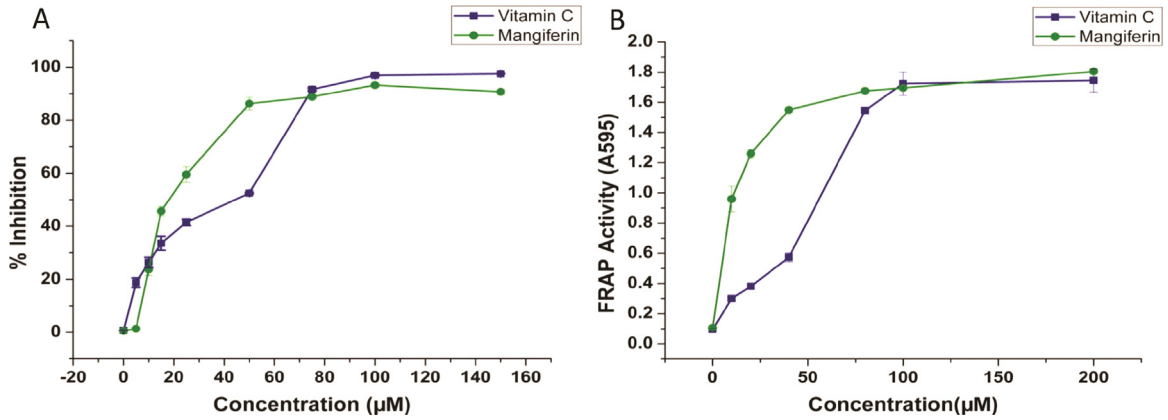

Fig. 1. Comparison between the antioxidant powers of mangiferin and Vit C in cell-free systems. (A) Represents the DPPH radical scavenging activities (expressed as % inhibition of control) of mangiferin and Vit C. (B) Shows the ferric reducing property of mangiferin and Vit C by FRAP assay. Vit C used as a positive control for both the experiments. Data represent the mean ± SD of 6 separate experiments. Closed circle: DPPH activity of mangiferin, closed square: DPPH activity of Vit C.

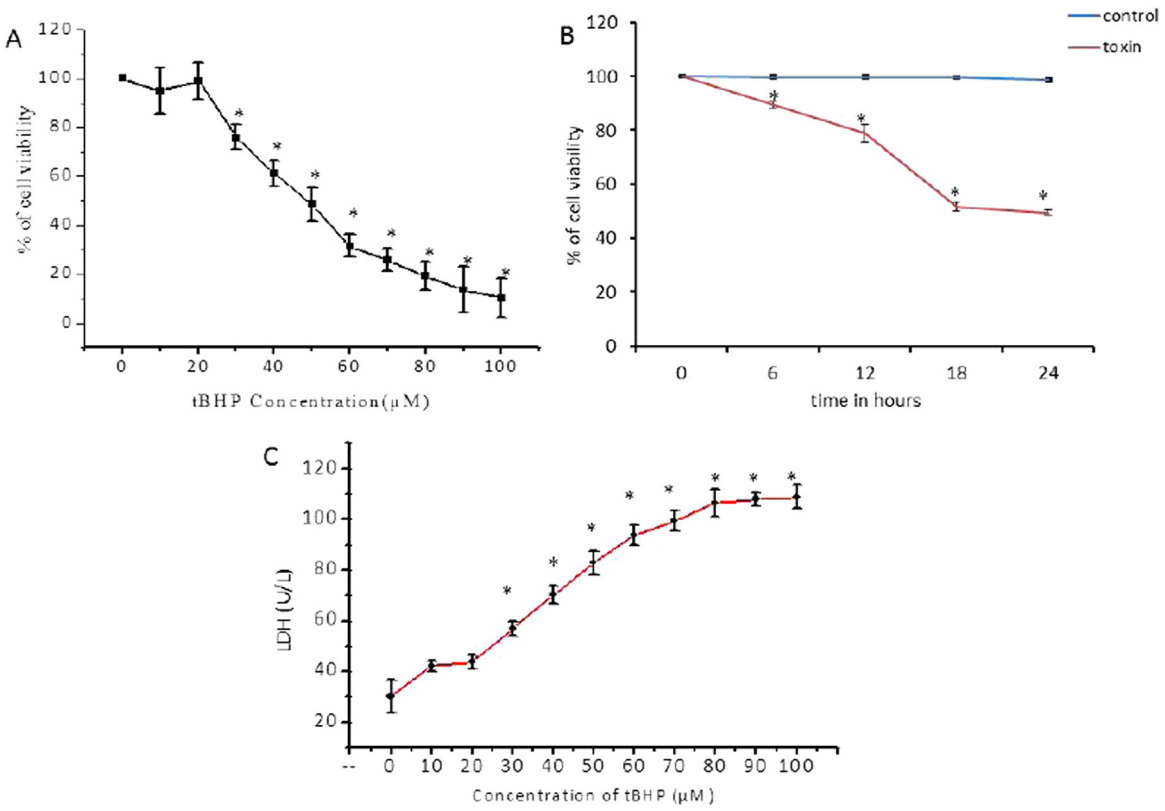

Fig. 2. The dose and time dependent effects on viability (expressed in % over control) and toxicity after tBHP exposure on NKE cells. (A) Effects of different concentrations of tBHP ranging from 10 μM to 100 μM, at a 10 μM interval on cells viability by MTT assay. (B) Time-dependent graph determining the time showing optimum toxicity of tBHP on NKE cells, for the period of 0–24 h in a 6 h interval. Each point represents mean ± SD, $n=6$ (number of plates). (C) Dose-dependent effect of tBHP on cellular toxicity as measured by LDH leakage assay [unit/litre]. Each column represents mean ± SD, $n=6$. "*" represents the significant difference between the normal control and tBHP exposed cells ($P^* < 0.05$).

18 h of its exposure. 2 h treatment of mangiferin prior to tBHP exposure was found to be effective against the oxidative insult. It was observed that treatment of 20 μM mangiferin exhibits only 10% cell death when exposed to 50 μM tBHP for 18 h (Fig. 3B). This experimental data was also compared with a standard potent antioxidant Vit C. It is observed that Vit C can also exhibit protective effect, but the effect of mangiferin is more pronounced (Fig. 3C). The same observation of more antioxidant potency of mangiferin was further confirmed by LDH cytotoxicity assay (Fig. 3D).

3.4. Mangiferin ameliorated oxidative stress and decreased lipid peroxidation in tBHP induced NKE cells

It is well documented that tBHP administration generates excess ROS production in various cells and tissues resulting in organ pathophysiology [55]. Moreover, so far, we had found that tBHP causes cytotoxicity and cell death in NKE cells, which was decreased on mangiferin administration. Thus, ROS could be the causal agent behind this devastating effect of tBHP. So to examine this, the level of ROS was measured in the control, tBHP and mangiferin treated cells. As presumed, the intracellular ROS levels in the tBHP exposed cells increased enormously compared to the control group and decreased significantly on treatment with mangiferin as shown in Fig. 4. It was found that at LC50 dose of tBHP, the cells faced intense oxidative injury as evident from the three-fold increment in the DCF fluorescence intensity which was resisted in the mangiferin treated group. Moreover, tBHP caused increase in lipid peroxidation in the NKE cells and that was resisted on mangiferin treatment (Fig. 4B).

3.5. Mangiferin restored the levels of cellular metabolites and antioxidant enzymes in tBHP induced cells

tBHP exposure caused a significant reduction in the level of reduced glutathione (GSH) and total thiols levels in association with an increased level of oxidized glutathione (GSSG). Treatment with mangiferin prevents the oxidation of GSH to GSSG via its antioxidant effect (Fig. 5A). Phase II antioxidant enzymes, the primary line of cellular defense in oxidative stress also decreased markedly in tBHP exposed cells, whose levels were significantly restored on mangiferin administration (Fig. 5B).

3.6. Mangiferin prevented the morphological alterations in the tBHP treated cells

External and internal morphological changes that occurred in the tBHP group of cells (due to oxidative stress induced by tBHP) was decreased on mangiferin treatment. There was a reduction in membrane blebbing resulting in decreased apoptotic bodies in the mangiferin treated tBHP exposed group (Fig. 6).

3.7. Mangiferin significantly decreased apoptosis induced by tBHP in NKE cells

Since the changes in morphology indicate that the damage caused by tBHP maybe through apoptosis, both Annexin V binding assay and DNA fragmentation assay was done to confirm the mode of cell death. The increase in the number of annexin V tagged apoptotic cells and the ladder formation in DNA fragmentation assay of the tBHP exposed cells, proved that the mode of tBHP

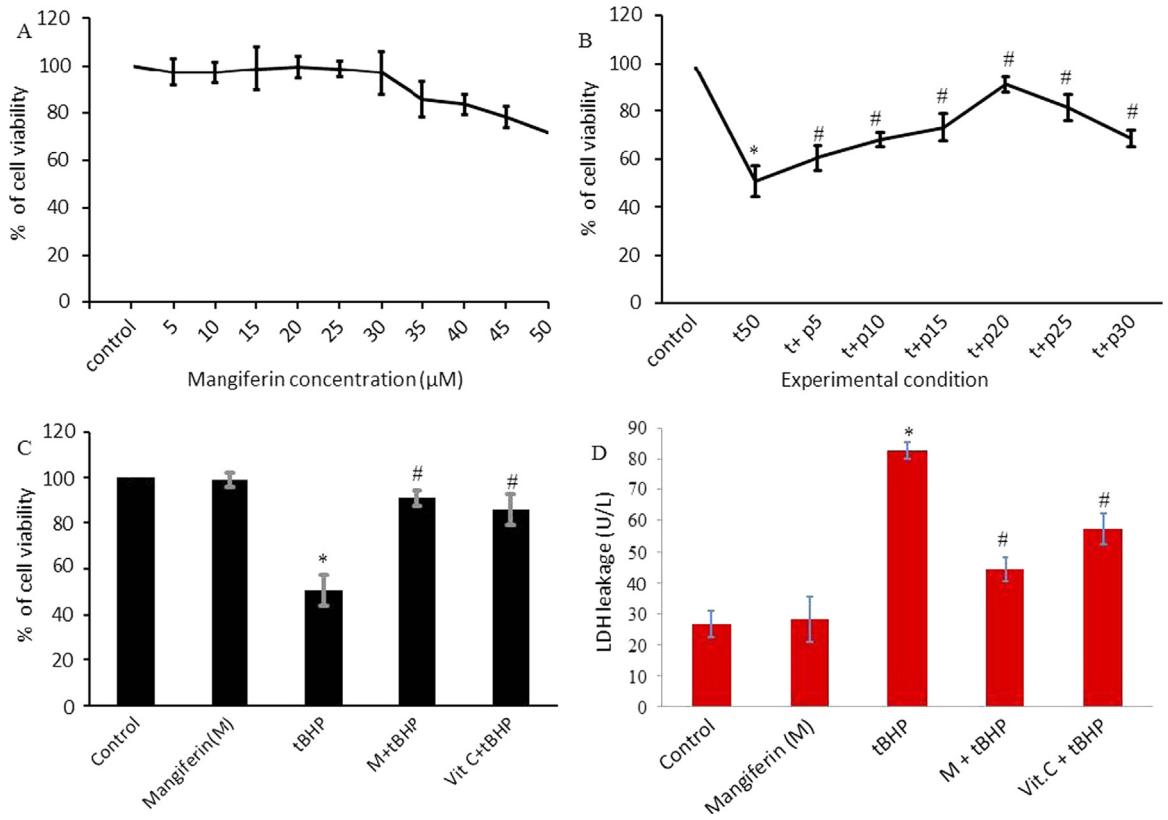

Fig. 3. Effects of mangiferin treatment on the NKE cell viability as well as cellular toxicity. (A) Effects of different concentrations of mangiferin ranging from 0–50μM, at an interval of 5 μM on NKE cells. (B) Protective effect of different concentrations of mangiferin (ranging from 0 to 30 μM, at a 5 μM interval) treatment 2 h preceding 50 μM tBHP exposure. (C) Figure showing the effect of tBHP intoxication, mangiferin treatment and Vit C treatment on NKE cells viability. (D) Comparison of cytotoxicity assays on tBHP, mangiferin and Vit C exposure with untreated control cells. Each point represents mean ± SD, $n=6$ (number of plates). "*" represents the significant difference between the normal control and tBHP exposed cells and "#" represents the significant difference between the tBHP exposed and tBHP exposed antioxidant treated cells ($P^* < 0.05$, $P\# < 0.05$).

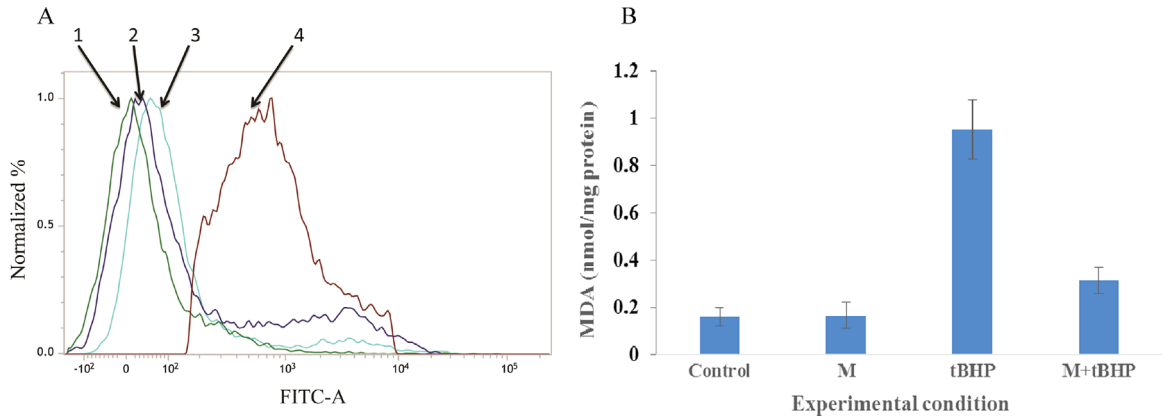

Fig. 4. (A) Effect of tBHP and mangiferin on ROS production in the kidney cells. DCFH-DA staining shows increased ROS production on 50 μM tBHP exposure (for 18 h) and decrease in ROS upon 20 μM mangiferin treatment (2 h) in the NKE cells. Intracellular ROS production was detected by changes in the fluorescence intensity of DCF, the oxidized derivative of DCF-DA via flow cytometry. 1: control untreated cells; 2: mangiferin treated cells; 3: tBHP intoxicated cells; 4: mangiferin treated tBHP exposed cells. (B) effect of tBHP and mangiferin on lipid peroxidation.

induced cell death is by the programmed cell death or apoptosis (Fig. 7A and B). In line with the previous observations, mangiferin treated cells exhibits no such significant indications of apoptosis.

3.8. *Decrease in mitochondrial membrane potential in tBHP injured cells is improved on mangiferin treatment*

Once the mode of cell death was confirmed to be apoptosis, we wanted to reveal whether it was mitochondrial or extra-mitochondrial pathway of apoptosis. A characteristic feature of the early stages of apoptosis is the disruption of active mitochondria if it follows the mitochondrial pathway of apoptosis [56]. So, we checked the integrity of the mitochondrial membrane by determining its membrane potential as mitochondrial damage includes alterations in the membrane potential and changes to its oxidation–reduction potential. The membrane-permeant JC-1 dye is commonly used to study mitochondrial health in apoptosis studies. We found a significant increase of green fluorescence and

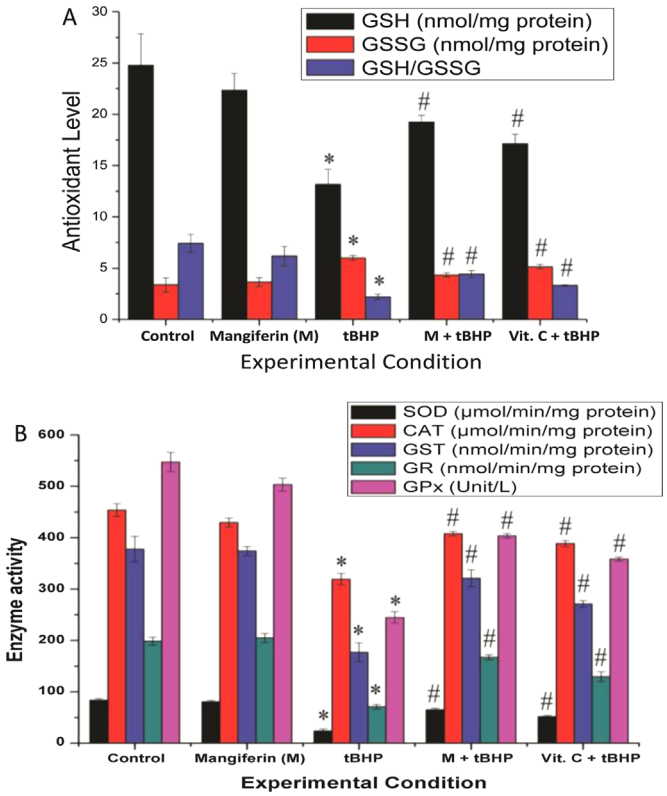

Fig. 5. (A) Effect of tBHP and mangiferin on Glutathione (GSH and GSSG), GSH to GSSG ratio, activities in NKE cells. (B) Effect of tBHP and mangiferin on the activities of phase II antioxidant enzymes in NKE cells. Each column represents mean ± SD, $n=3$. "*" represents the significant difference between the normal control and tBHP exposed cells and "#" represents the significant difference between the tBHP exposed and tBHP exposed antioxidant treated cells ($P^* < 0.05$, $P\# < 0.05$).

consequent decrease in the red fluorescence in the MMP of the tBHP treated cells when compared with control (confirmed by the decreased binding of cationic JC1 dye to the mitochondrial membrane, Fig. 8).

3.9. Modulation of Bcl2 family proteins

An increased Bax/Bcl2 ratio is a primary hallmark of apoptosis. Eventually, while studying some proapoptotic and antiapoptotic proteins to reveal the apoptotic pathway, we intended to observe the effect of tBHP and mangiferin on the Bax/ Bcl2 ratio. It was observed that the tBHP exposed cells had highly increased Bax/ Bcl2 ratio compared to the untreated NKE cells and mangiferin potentially decreased the Bax/ Bcl2 ratio in NKE cells, irrespective of tBHP exposure (Fig. 9A).

3.10. Mangiferin attenuate the tBHP induced mitochondrial and extra mitochondrial pathways of apoptosis

Oxidative stress is directly linked to the dysfunction of mitochondria. As it has been stated above, tBHP causes a decrease in mitochondrial membrane potential (MMP) and mangiferin almost nullifies the effect of tBHP. Disruption of MMP causes the release of cytochrome C in the cytosol due to the decoupling of the respiratory chain and subsequently cytochrome C activates the caspases that lead the cells to undergo apoptosis [26]. In this study, we have examined the signaling mechanism, activated by ROS. By evaluating different stress responsive protein in the whole cell extract of the treated NKE cells, we found that tBHP exposure makes an elevation of the level of activated caspase 8. In the downstream we observed an upregulation of t-Bid and subsequently increased Bax and cytochrome C expression. We further evaluated some proteins that are known to be regulated by cytochrome C. We found an increased level of expression of Apaf-1 and in the downstream activation of different proapoptotic caspases like caspase 9 followed by caspase 3.

Previously we observed that tBHP caused DNA fragmentation. So, we intend to check the activity of PARP as a confirmation of DNA fragmentation. It is observed that in tBHP treated cells there is an increased level of PARP cleavage.

In line with our previous data, we observed that mangiferin treatment significantly regulates the function of these pro-apoptotic proteins as confirmed by their decreased expression (Fig. 9B). In mangiferin treated cells, the level of total PARP is properly maintained.

3.11. Involvement of PI3K

Phosphatidylinositide 3-kinases (PI3K) are a class of enzymes, regarded as "well wisher" molecules of any normal cell. These are involved in cellular growth, differentiation, motility, survival, etc [57]. In this present study, we observed by immunoblotting analysis that the level of PI3K was decreased in the tBHP exposed group and it got increased in cells exposed to both tBHP and mangiferin together. To confirm that mangiferin shows its protection via the activation of PI3K, NKE cells were pretreated with the kinase inhibitor for 1 h at a concentration of 10 μM [54,58,59], and then cells were treated with 20 μM mangiferin before tBHP exposure. After treatment, the cell viability was determined. As shown in Fig.10A, pretreatment of cells with a pharmacological inhibitor of PI3K (LY294002) effectively reduced the cell viability to a great extent. This indicates PI3K as the molecule playing a pivotal role in mangiferin's protective activity on NKE cells.

3.12. Effect of mangiferin on Nrf2 nuclear translocation and antioxidant enzyme expression

Then we studied the expression level of some anti-oxidant genes that are known to be expressed by the activity of PI3K. We observed a reduced expression of Nrf-2 in the tBHP exposed group and increased level of expression in mangiferin and tBHP co-exposed group. Besides, in the downstream of Nrf-2, we found an elevated expression of antioxidant molecules, HO-1, and SOD-2 in mangiferin and tBHP coexposed cells than the cells exposed only with tBHP. However, pretreatment of NKE cells with PI3K inhibitor (LY294002) showed that the nuclear translocation of Nrf2, which was elicited on mangiferin treatment, got downregulated (Fig.10A).

3.13. Alterations in Akt-GSK3β pathway

Since we got an increased expression of PI3K, we also wanted to study the expression level of other proteins related to the activation of PI3K. PI3K generally acts through Akt phosphorylation and mTOR to regulate the cell cycle. The level of these proteins decreased in the tBHP exposed cells than the control cells. We observed a significant increase in the phosphorylated Akt and expression level of mTOR, in the downstream of pAkt in the mangiferin plus tBHP group. In the downstream of pAkt, decreased level of nuclear NFκB was found in the cells exposed to the only tBHP and an increased expression level of nuclear NFκB in cells exposed to both mangiferin and tBHP was found. Again, the level of BAD was also found to be decreased on mangiferin treatment possibly due to its phosphorylation by activated AKT. We then treated the cells with the PI3K inhibitor (LY294002). As shown in (Fig.10B), mangiferin induced phosphorylation of AKT was blocked by pretreatment of cells with LY294002.

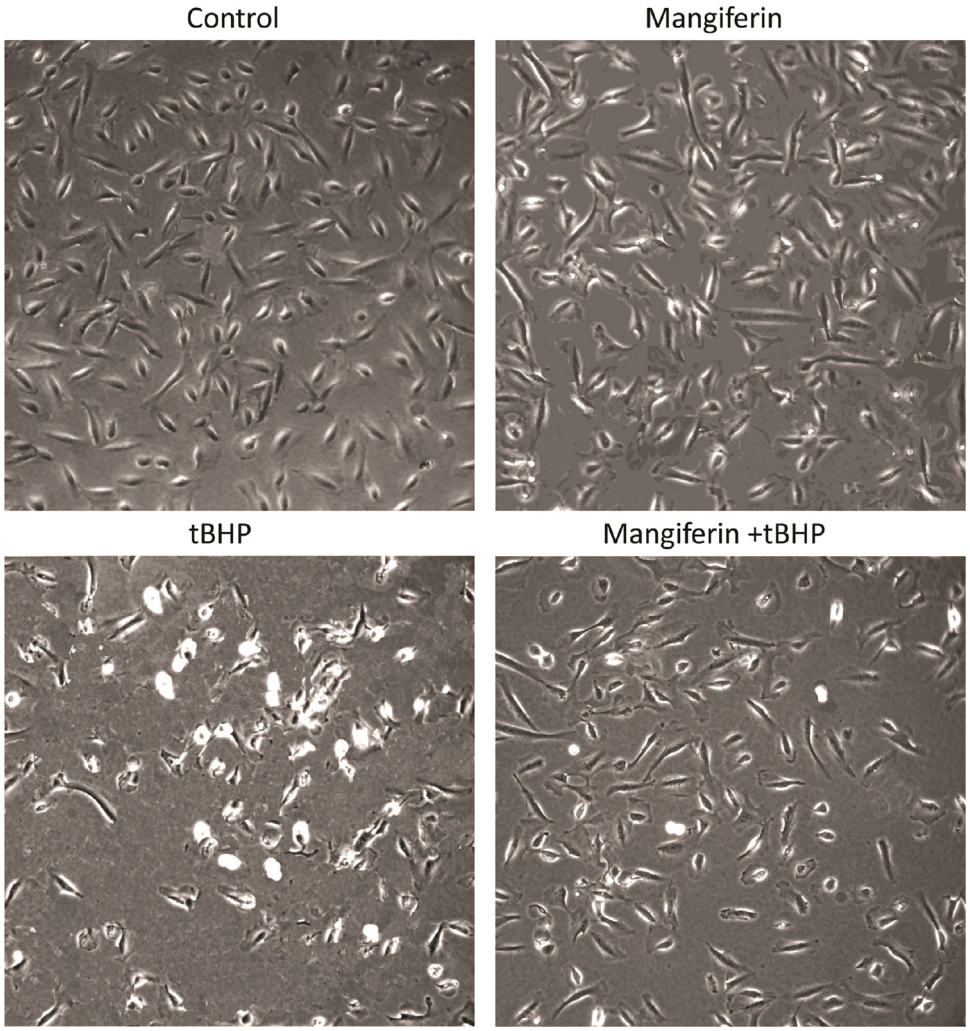

Fig. 6. Phase contrast micrographs (100 ×) showing external morphological changes in cells. tBHP exposed cells show blebbing, indicating cells with altered membrane morphologies. Mangiferin treatment decreases such morphological changes associated with tBHP administration.

Next we studied the effect of tBHP on cell cycle proteins. We observed a down regulated expression of cyclin D1 in tBHP exposed cells compared to the untreated cells and a reversal to this was observed when the tBHP exposed cells were treated with mangiferin. Upstream of cyclin D1, pGSK 3β level was also determined and an elevated expression was observed in tBHP exposed cells. On the other hand, a protective effect was observed when the cells were treated with the flavonoid (Fig.10B).

3.14. Mangiferin attenuate tBHP induced cell cycle arrest

As discussed earlier, tBHP downregulates the expression of cyclin D1, which is critical for the G1/S transition in the cell cycle. From flow cytometric analysis, it was observed that exposure to tBHP significantly arrested the cell cycle at G0/G1 phase. Mangiferin pretreatment significantly attenuated the alteration induced by tBHP exposure (Fig.11).

4. Discussion

Research around the globe suggests that similar to other organs, a critical level of ROS is truly necessary for normal kidney homeostasis [60]. However abnormal elevation in intracellular ROS level leads to the oxidative stress phenomenon, leading to a disturbance in the normal redox level of the cell [60,61]. This results in toxic effects on the cellular macromolecules viz. carbohydrates, proteins, and lipids [62].Oxidative insult can also potentially modulate the activity of a number of signaling molecules which ultimately leads to cell death [63]. Endogenously a cell can produce various antioxidant molecules in response to oxidative stress in order to maintain the optimum intracellular ROS level and these molecules have the potential to scavenge the reactive molecules by transferring electrons [64]. However, when an external stimulus causes an abnormal elevation of oxidative species, it nullifies the intracellular antioxidants and causes deleterious physiological changes [65,66]. In such pathophysiological condition, external administration of antioxidants becomes essential to combat the stress successfully that evolved. Such antioxidant molecules can be of various chemical natures and has the capability to modulate a wide array of signaling molecules thus showing fruitful results in most of the cases [51,67,68].

Dietary isoflavone mangiferin is a potent antioxidant, which has both the properties of scavenging free radicals and modulating the gene regulation [69]. In our study, we also confirmed the potency of mangiferin as an antioxidant by comparing its antioxidant power with Vit C. On performing the antioxidant assays in cell-free system (DPPH and FRAP assays), the antioxidant activity of mangiferin was found to be even better than the activity of Vit C at lower doses. To further corroborate the oxidative stress mitigating power of mangiferin compared to Vit C, we performed the cell viability and LDH cytotoxicity assays on NKE cells treating them

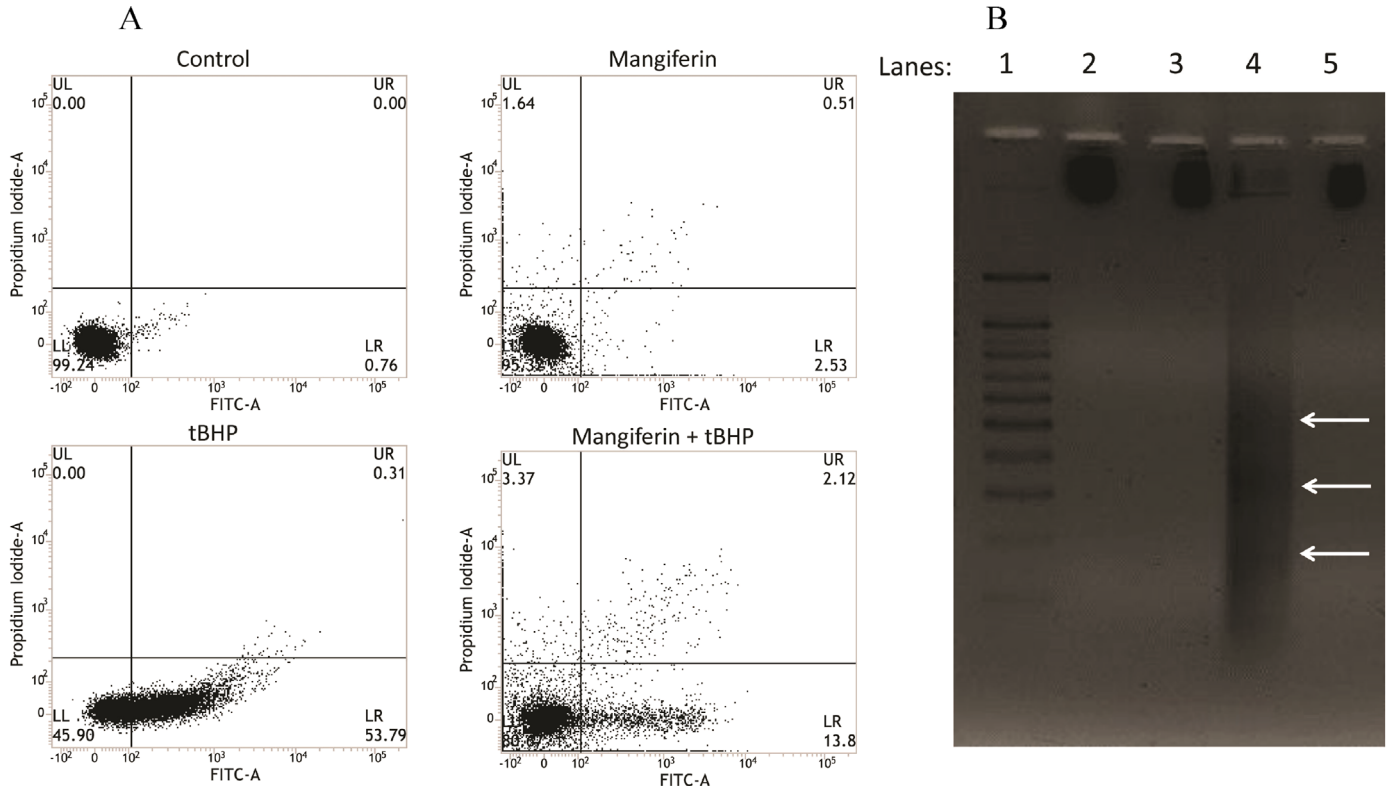

Fig. 7. (A) Detection of tBHP induced apoptosis, anti-apoptotic effect of mangiferin and effect of both together in NKE cells via Annexin V-FITC staining. The NKE cells were incubated separately with desired molecules (tBHP, mangiferin and both) followed by staining with Annexin V-FITC (FL1-H) and then the cells were flow cytometrically analyzed. Dual parameter dot plot of FITC-labeled 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. (B) Study of DNA fragmentation on agarose/ethidium bromide gel. Isolated DNA from untreated and treated cells was loaded onto 1% (*w*/*v*) agarose gels. Lane 1: marker (1 kb DNA ladder); Lane 2: DNA isolated from normal untreated NKE cells; Lane 3: DNA isolated from mangiferin treated cells; Lane 4: DNA isolated from tBHP intoxicated NKE cells; Lane 5: DNA isolated from cells in which mangiferin was treated before tBHP administration.

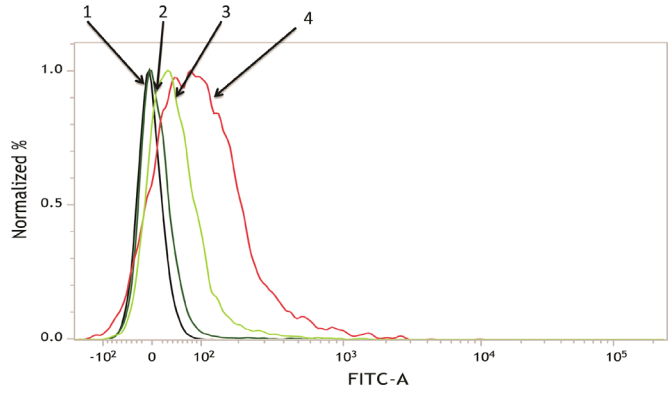

Fig. 8. Effect of mangiferin on tBHP induced decrease in mitochondrial membrane potential in NKE cells as shown by the monomeric green fluorescence of the JC 1 dye. Data are representative of three independent experiments. 1: Control untreated cells; 2: mangiferin treated cells; 3: tBHP intoxicated cells; 4: Mangiferin treated tBHP exposed cells.

with both the antioxidants individually in association with tBHP exposure. The results showed that the mangiferin is better than Vit C in ameliorating tBHP induced oxidative and cytotoxic injury. Moreover, the exposure of mangiferin significantly reduces the percentage of cell death and LDH leakage of the NKE cells upon tBHP administration. Fig. 12 shows the intrinsic mechanism by which mangiferin exhibits it's antioxidant potential.

A number of scientific reports suggest the adverse role of tBHP both *in vivo* and *in vitro* indicating tBHP as a causal agent of oxidative stress [70]. We performed a dose-dependent effect on cell viability and cytotoxicity using various concentrations of tBHP ranging from 0 to 100 μM to find the dose of tBHP causing 50% cell death. The LC_{50} was found to be 50 μM tBHP. Exposure to tBHP causes an abnormal elevation of intracellular ROS. So, the level of endogenous ROS was determined which agreed with the previous reports [71]. We found an abrupt increase of ROS production by increased DCF production on subjecting the cells to 50 μM LC_{50} dose of tBHP. Accumulation of ROS causes a disturbance in normal antioxidant homeostasis [72]. So, the endogenous levels of the different nonenzymatic and enzymatic cellular antioxidants were measured next and found that those levels were significantly reduced by tBHP. On the other hand, mangiferin treatment helped in combating ROS and increasing the decreased antioxidant levels. It also helped the cells to maintain the normal level of different intracellular antioxidants like SOD, CAT, and GST. Besides, this molecule prevented lipid peroxidation and LDH leakage from the renal cells. Increase in lipid peroxidation and LDH leakage clearly indicates a reduction in the percentage of cell viability [73]. As mangiferin treatment can prevent the lipid peroxidation and LDH leakage, it could reduce the percentage of tBHP induced cell death.

Further, we wanted to check if there were any structural alterations in the NKE cells due to tBHP exposure. So we studied phase contrast micrographs that also indicate a change in cell morphology. In the case of tBHP exposed cells, plasma membrane was found to be disrupted, and many apoptotic bodies were observed throughout the culture dishes. In line with earlier evidence, we observed that in mangiferin treated cells, the outer cell morphology remained normal and the number of apoptotic bodies got reduced. Though isoflavones are harmful in some cases but 20 μM mangiferin is not found to be harmful in respect to cellular

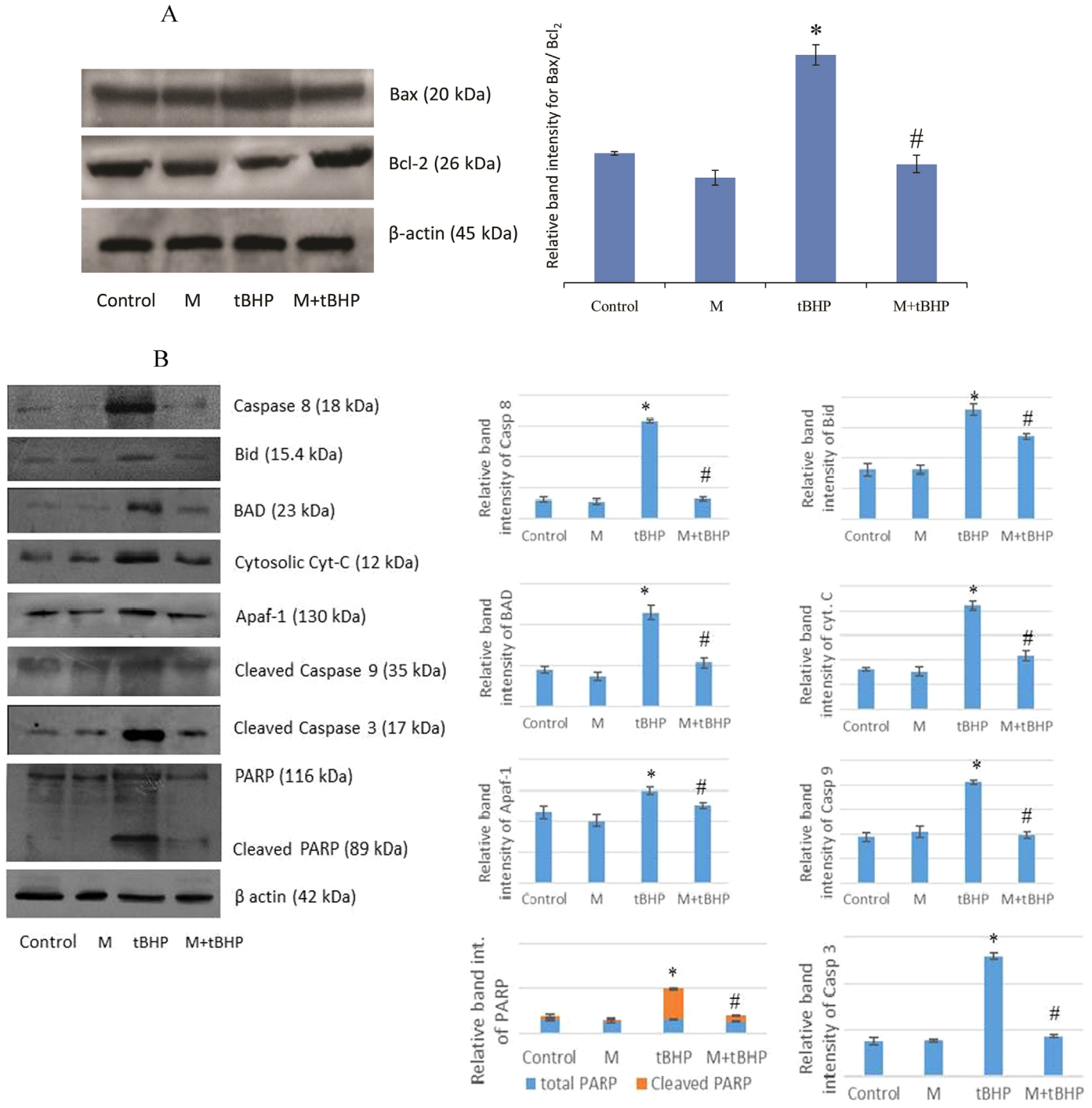

Fig. 9. (A) Immunoblot analysis of Bax and Bcl2 in response to tBHP and mangiferin exposure, in NKE cells. (B) Immunoblot analysis of Caspase 8, tBid, Bax, cytochrome C, Apaf-1, Caspase 9, Caspase 3 and PARP. NKE cells were exposed to tBHP (50 μM) and mangiferin (20 μM) according to the defined protocol. β actin was used as an internal control Control: untreated cells, M: exposed to mangiferin, tBHP: exposed to tBHP for 18 h, M+tBHP: Mangiferin treated for 2 h followed by 18 h of tBHP exposure. For inhibitor studies, M+T+I: cells were treated with LY294002(I) follwed by Mangiferin(M) and Tbhp(T). All data are mean ± SD, of 3 independent experiments and were analyzed by one-way ANOVA. "*" represents the significant difference between the normal control and tBHP exposed cells and "#" represents the significant difference between the tBHP exposed and tBHP exposed antioxidant treated cells ($P^* < 0.05$, $P\# < 0.05$).

morphology when compared to the untreated cells. Viability studies of cells treated solely with mangiferin also confirm the same.

AnnexinV mediated apoptosis assay and DNA fragmentation assay were then performed to confirm the mode of cell death. Both increased Annexin V fluorescence and DNA ladder formation on agarose gel confirm the apoptotic mode of tBHP induced cell death. The ongoing research encircling oxidative stress have suggested that mitochondrial damage, elevated ROS level and the programmed cell death are very interrelated since most of the time elevated ROS leads to mitochondrial damage and finally cell death by apoptosis [56]. Disruption of the transmembrane potential is a clear indication of apoptotic cell death. In this study, we found both elevated ROS level, increased lipid peroxidation and apoptosis and thus it was essential to monitor the mitochondrial condition of tBHP exposed NKE cells. Thus to check the mitochondrial health, the integrity of its membrane was examined by measuring the MMP with the help of JC1 dye. There was a huge loss of MMP in the tBHP group. Mangiferin treatment could

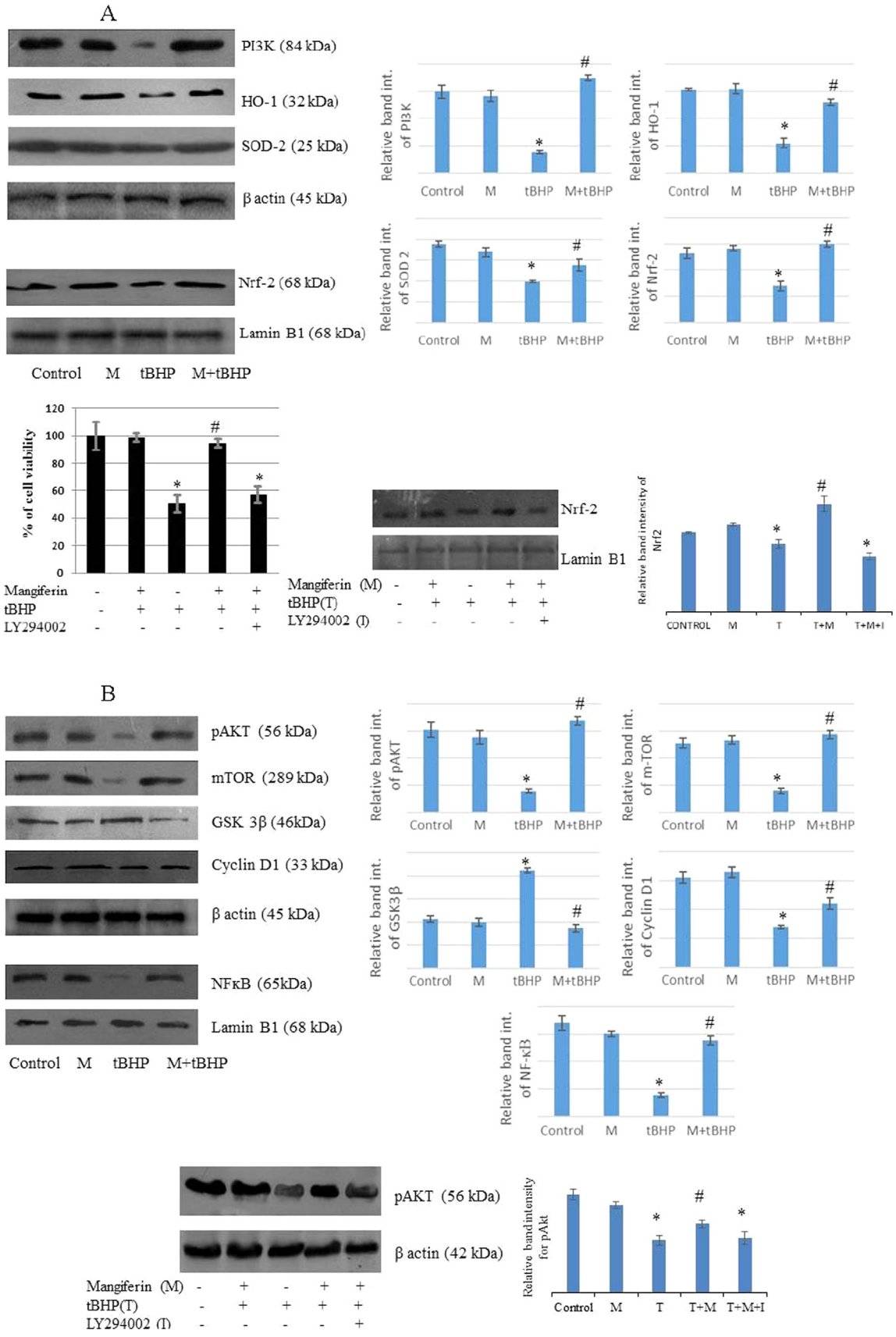

Fig. 10. (A) Immunoblot analysis evaluating the expression of PI3K, Nrf-2, HO-1, SOD-2 signaling molecules. Pretreatment of cells with pharmacological inhibitor of PI3K (LY294002) shows decreased cell viability and decreased expression of Nrf-2 in the nucleus, even on mangiferin exposure after tBHP toxicity in NKE. (B) Immunoblot analysis of pAkt, mTOR, NF-κB, cyclin D1, GSK 3β, in different subgroups of NKE cells. LY294002 pretreated mangiferin and tBHP exposed cells showed decreased expression of pAkt that was increased on mangiferin exposed tBHP intoxicated cells. β actin and Lamin B1 was used as an internal control. Control: untreated cells, M: exposed to mangiferin, tBHP: exposed to tBHP for 18 h, M + tBHP: Mangiferin treated for 2h followed by 18 h of tBHP exposure, M + T + I: cells were treated with LY294002 follwed by Mangiferin and tBHP as stated above. 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 tBHP exposed cells and "#" represents the significant difference between the tBHP exposed and tBHP exposed antioxidant treated cells ($P^* < 0.05$, $P\# < 0.05$).

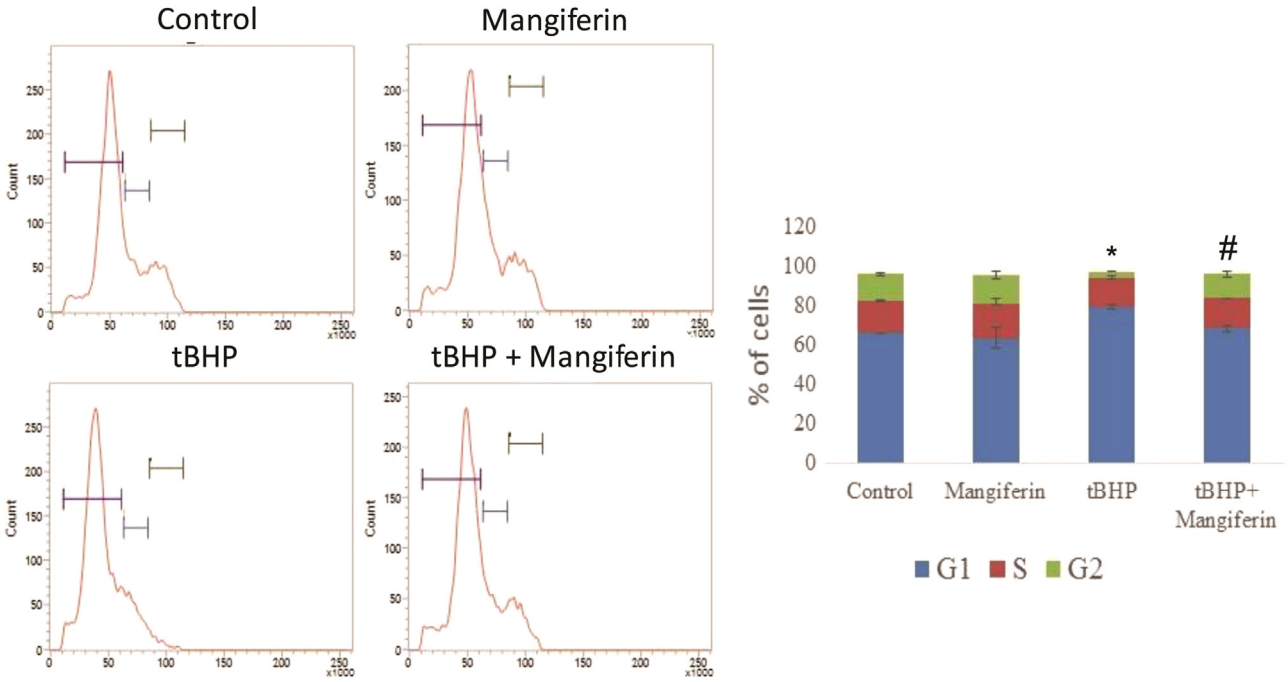

Fig. 11. Cell cycle analysis shows that NKE cells when exposed to tBHP showed cell cycle arrest on G1 phase. Pretreatment with Mangiferin accelerated cell cycle progression by inducing the expression of cyclin D1.

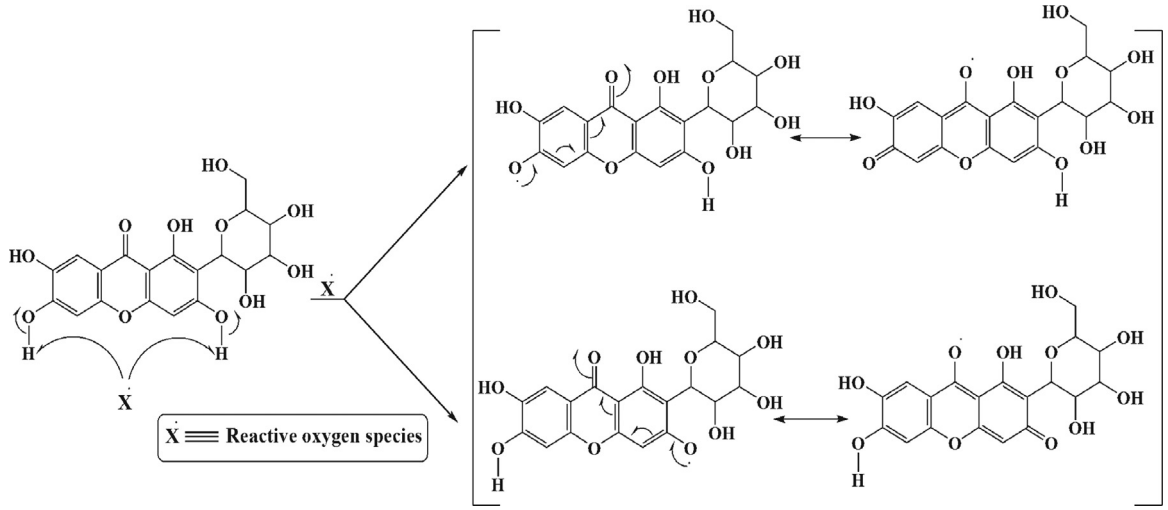

Fig. 12. Possible mechanism of free radical scavenging activity by mangiferin.

significantly inhibit the apoptotic cell death, decrease the elevated ROS and lipid peroxidation. Mangiferin exposure also increased the MMP of the cells by reverting them back from pathophysiological state to nearly, if not completely, normal physiology. Thus, all the results so far indicate that the mangiferin can combat the oxidative stress and related injuries induced by tBHP on renal cells. So, to elucidate the unknown mechanisms by which tBHP causes harm and mangiferin potentially shield this deleterious effect of tBHP in NKE cells, immunoblot analysis were carried out. Immunoblot analysis helped to identify the key molecules involved in the signaling cascades.

tBHP can cause cell death by two pathways: (1) by mediating ROS, through mitochondrial dysfunction and (2) by activating caspase 8 through an extrinsic mechanism. ROS accumulation by tBHP causes an activation of the proapoptotic protein BAD, which then activates a series of molecules including initiator and effector caspases leading to apoptotic cell death by mitochondrial pathway [74].

Mangiferin protects the cells from tBHP induced activation of caspases and imbalance in the Bax/Bcl_2 ratio. In addition, mangiferin significantly up-regulates survival proteins PI3K and pAkt in response to the induced cytotoxicity. To examine if tBHP induced apoptosis solely follows mitochondrial pathway or the extra-mitochondrial pathway of apoptosis also has a role, immunoblot analysis of Caspase 8 was performed. An up-regulated response of Caspase 8 was found, indicating the involvement of extra-mitochondrial pathway too. Again it was found that there was an increased expression of Bid, which then may get converted into its truncated form tBid by Caspase 8 [38]. In downstream, we also observed an upregulated pro-apoptotic response of Bcl2 family

protein Bax and cytosolic level of cytochrome C. Bax then coupled with the mitochondrial membrane and formed an oligomeric pore in the mitochondrial membrane. It causes the disruption of mitochondrial membrane potential and hence the release of cytochrome C in the cytosol. Bax and the cytosolic cytochrome C have the potential to activate different caspase family proteins [75,76]. Existing reports suggest that during the presence of apoptotic signal the cytosolic Cyt-C along with dATP binds with the Apaf-1 to form an oligomeric apoptosome [77]. This complex binds to procaspase 9 and cleaves it to form activated caspase 9. Our results also supported these findings. Caspase 9 is an initiator caspase, and once it gets activated, it activates caspase 3 and this causes the proteolysis of PARP. The proteolysis of PARP is responsible for the induction of apoptosis and by preventing the cell survival mediated by DNA repair and also conserves energy to prevent necrosis [78]. We also found increased levels of Caspase 9, Caspase 3 and cleaved PARP in our studies.

Our further investigation on the mode of apoptosis showed that the expression of pAkt, induced by its precursor molecule PI3K, gets down-regulated in tBHP exposed cells and the expression was found to be higher in case of mangiferin treated tBHP exposed cells. pAkt causes the phosphorylation of BAD, but when pAkt is suppressed, BAD remains dephosphorylated. Dephosphorylated BAD forms a heterodimer with Bcl2 and displaces Bax. This event leads to an elevated Bax to Bcl2 ratio, and it leads to apoptosis.

Existing literature suggest that ROS causes an upregulation of GSK 3β, and it can inhibit the activity of cyclin D1 [79]. So examining their expression we found a down-regulation of cyclin D1 and up-regulation of GSK 3β in tBHP exposed cells, and the reverse effect was observed with mangiferin treatment. This event suggests that possibly there is cell-cycle arrest in the G1 phase in tBHP exposed cells, and fortifying effect of this is observed with mangiferin treatment. In the case of mangiferin treatment, GSK 3β gets inhibited and hence the function of cyclin D1 is retained.

Here we propose a role for PI3K/AKT as a possible regulator of cell survival after mangiferin exposure. In the present study, we found pretreatment of mangiferin upregulates the level of PI3K in tBHP exposed cells. Hence, it could also possibly up-regulate the expression of Nrf-2. Nrf-2 is a basic leucine zipper and can regulate the expression of different antioxidant enzymes. However, it is also observed that pharmacological inhibitor of PI3K (LY294002) blocks the expression of the downstream signaling pathway by suppressing the expression of Akt and nuclear translocation of Nrf2. From the above observation, we can conclude that the cytoprotective action of mangiferin on the tBHP exposed cells is PI3K dependent.

In addition to the above findings, we also observed an elevated expression of HO-1 and SOD-2 [80]. Literature suggests that Nrf-2 has the potential to up-regulate both these enzymes. Moreover, HO-1 can also up-regulate the expression of SOD-2 [81].

On the other hand, as we have found an increased expression of mTOR and NF-kB, these two proteins may get activated by the activity of pAkt. mTOR (mammalian target of rapamycin) is a serine-threonine kinase that promotes cell growth and proliferation [82]. Thus, mangiferin hinders cellular apoptosis and encourages cell growth. Summary of the up regulation and down regulation of different molecules discussed so far on tBHP and mangiferin administration are schematically represented in Fig. 13.

5. Conclusion

In conclusion, our study suggests that tBHP induced cytotoxicity caused apoptotic cell death in normal kidney epithelium cells via oxidative stress-induced cell signaling pathways. In contrast, mangiferin has the potential to maintain normal cellular homeostasis by resisting the detrimental effect of tBHP and promoting cell proliferation by trigerring the expression of PI3K and its downstream molecules. Combining, we say that further research may establish this polyphenolic bioactive flavonoid, mangiferin, as a possible candidate for the treatment of oxidative stress associated renal complications in future.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.01.011.

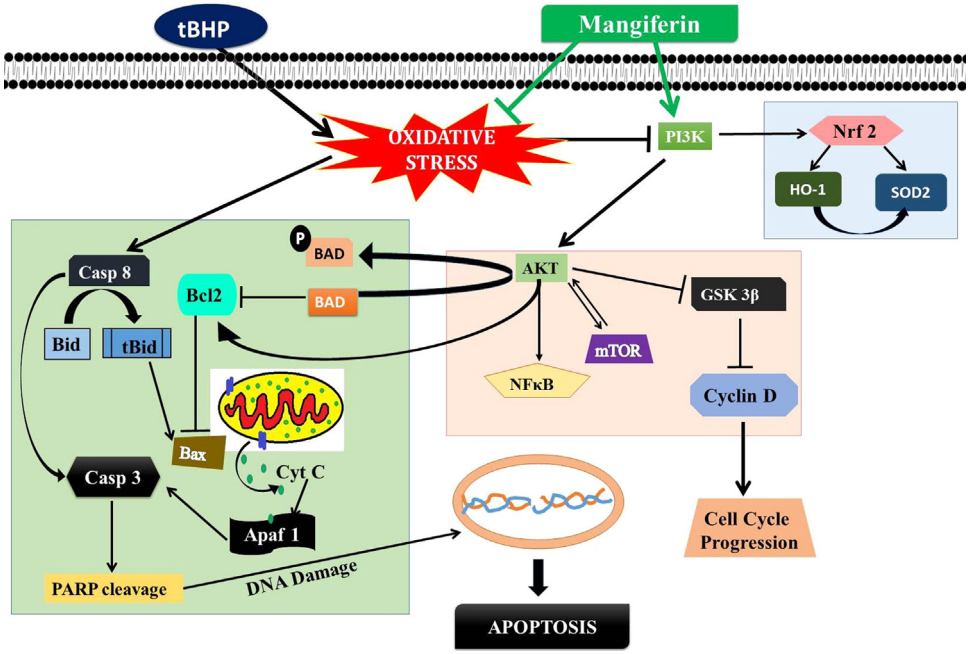

Fig. 13. The differential expressions of various signaling molecules due to mangiferin treatment and tBHP exposure respectively.

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