



# *Phlogacanthus thyrsoiflorus* Nees. modulates hepatic and renal apoptosis via attenuation of oxidative stress in alloxan-administered mice

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## Abstract

An endeavor has been made in this study to assess the effect of *Phlogacanthus thyrsoiflorus* on hyperglycemia-induced oxidative stress along with apoptosis in liver and kidney of alloxan-administered mice. To analyze the effect of *Phlogacanthus thyrsoiflorus* on oxidative stress in kidney and liver of diabetic mice. Furthermore, we have also examined the effect of *Phlogacanthus thyrsoiflorus* on apoptosis in diabetic mice. Following preparation of methanolic flower extract (MFE), preliminary phytochemical screenings and acute toxicity test were carried out for MFE. Lipid peroxidation and protein carbonyl assays were determined to check the MDA level and oxidative damage in tissues of groups normal control mice (NCM), diabetic control mice (DCM), ascorbic acid-treated diabetic mice (D + AA), and MFE-treated diabetic mice (D + MFE). Histological and ultrastructural studies were conducted to evaluate any changes in tissues as well as sub-cellular organelles. The effects of MFE on caspase 3 and Bcl-2 expression in alloxan-induced diabetic mice were studied and compared against the diabetic control group of mice. Upon treatment with MFE, the diabetic mice manifested a notable depletion of malondialdehyde (MDA) and protein carbonyl levels. The ultrastructural studies divulged the capability of MFE to reinstate morphological and cellular alterations as contemplated in alloxan-induced diabetic mice. On apoptosis, the effect of MFE showed the downregulation of cysteine-dependent aspartate specific protease (caspase) 3, whereas upregulation of B-cell lymphoma-2 (Bcl-2) protein and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) assay illustrated a few apoptotic cells. It can be put forward that methanolic flower extract of *Phlogacanthus thyrsoiflorus* treatment can exert hepatoprotective and nephroprotective effect via regulating hyperglycemia-induced oxidative stress and apoptosis in alloxan-administered diabetic mice.

**Keywords** *Phlogacanthus thyrsoiflorus* · Methanolic flower extract · Alloxan · Hyperglycemia · Oxidative stress · Apoptosis

## Introduction

Diabetes mellitus is expanding to an alarming epidemic level of today's century (Shaw et al. 2010). By looking into the rapid increase of diabetes, it is predicted that the rate will be almost doubled by 2030 (Wild et al. 2004). Diabetes is clinically examined as hyperglycemia due to its persistent and/or insulin inadequacy (Mathis et al. 2001). On the other

hand, it is believed that oxidative stress has an important role in the development of various complications in diabetes (Pham-Huy et al. 2008). Elevation of ROS level in diabetes may be due to increase in destruction or/and decrease in the production by superoxide dismutase (SOD), catalase (CAT—enzymatic/non-enzymatic), and glutathione peroxidase (GSH-Px) antioxidants (Lipinski 2001). Several studies have concluded that there is an interconnection between diabetes and oxidative stress and oxidative stress is one of the vital contributors in the growth and advancement of diabetes (Maritim et al. 2003; Wright et al. 2006). Mitochondrial respiratory chain reaction and other operations such as glucose autoxidation, glycosylation, glucosamine, and polyol pathways enable ROS fabrication that eventually supplements the intracellular ROS level (Brownlee 2001; Giacco and Brownlee 2010), resulting in diminution

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of antioxidant defenses (Bandeira et al. 2013; Pitocco et al. 2013) and aggregation of large amounts of ROS (Rahman 2007; Pizzino et al. 2017) that lead to oxidative stress under diabetic conditions (Inoguchiet al. 2000; Sindhu et al. 2004). Increased accumulation of ROS can change the structure and function of biomolecules like proteins, membrane lipids, and nucleic acids with significant repercussions to cell potentiality (Jackson and Bartek 2009). Oxidative stress also persuades DNA destruction at basic sites (AP sites), and single-strand breaks and also double-strand breaks that oxidize DNA bases (Moreli et al. 2014); when DNA destructs outstrips the cellular extent to rehabilitate it, aggregation of mistakes can inundate the cell resulting in apoptosis.

India has been the home to several indigenous methods or alternatives for the treatment and management of several diseases since the sixth century (Kashikar and Tejaswita 2011). The excessive presence of free radicals in the body led to the cause of diseases such as rheumatoid arthritis, cancer, and diabetes; therefore, the use of natural compounds present locally is believed to be efficacious in scavenging these free radicals and thus ameliorating the disease processes. A herbal plant named *Phlogacanthus thyrsoiflorus*, commonly called titaaphul, chuhai, and dieng-soh, is a shrub of Acanthaceae family, and is one of such plants that have the potential scavenging property to scavenge these free radicals present in the body, hence proving it to be a very good medicinal herb (Jaiswal 2010). But as time passed, scientists failed to establish a report on its efficiency for treatment and, soon enough, its importance faded away. The abovementioned medicinal properties possessed by this medicinal herb do help us in considering this herb to undergo a systematic study.

In our past studies, we have reported that the shrub *Phlogacanthus thyrsoiflorus* has a protective effect against hyperglycemic, hyperlipidemic, and antioxidative properties in liver and kidney tissues of alloxan-induced diabetic mice (Bora et al. 2018a, b). It is also notable that hyperglycemia-induced ROS generation has consequences in the etiology of diabetic complications and results in apoptosis of cells. Hence, the current research work was framed to analyze the effect of *Phlogacanthus thyrsoiflorus* on oxidative stress in kidney and liver of diabetic mice. Furthermore, we have also examined the effect of *Phlogacanthus thyrsoiflorus* on apoptosis in diabetic mice.

## Materials and methods

### Chemicals

Alloxan, thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), dichlorofluorescein (DCF), 20,70-dichlorofluorescein

diacetate (DCFH-DA), 3,30,5,50-tetramethylbenzidine (TMB), diethylenetriaminepenta-acetic acid (DETAPAC), 40,6-diamidino-2-phenylindole (DAPI), and anti-caspase 3 antibody were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Bovine serum albumin (BSA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), sodium dodecyl sulfate (SDS), and anti-Bcl-2 antibody were obtained from Merck, India. Anti-β-actin and anti-caspase 9 antibodies were procured from Abcam, Cambridge, UK. The in situ cell death detection kit and fluorescein for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay were from Roche, Switzerland. The other reagents used for the study were purchased from Sisco Research Laboratory, Hi-Media, and SD Fine-Chem Ltd., India.

### Plant material

*Phlogacanthus thyrsoiflorus* flowers were collected during the winter season from Assam, India (Voucher No: 12055). The sample specimen was authenticated by Dr. P.B. Gurung Curator Herbarium, Department of Botany, NEHU, Shillong, Meghalaya.

### Methanolic extract preparation

The flowers were collected, separated, weighed, washed, and then subjected to shade-drying. The dried sample was then powered, homogenized, and extracted repeatedly with 10 × volume of methanol-aqueous solution (4:1) (Harborne 1998). The obtained dried mass was stored at – 20 °C to conduct all the experiments.

### Phytochemical screening

The MFE was subjected to qualitative phytochemical analysis for flavonoids, tannins, phenols alkaloids, and saponins as per the standard methods (Wani et al. 2012).

### Test animals

For this study, Swiss albino mice (Balb/C strain) were used, and healthy ones were chosen, weighing between 25 and 30 g. The mice were maintained with controlled temperature of 22.2 °C and kept under a light/dark cycle of approximately 12 h in a room; during this duration, they were fed only with standard laboratory feed and water ad libitum. Institutional Ethics Committee (IEC) guidelines were followed during the conduct of experiments. All institutional and national guidelines for the care and use of laboratory animals were followed.

## Acute toxicity study

In the acute toxicity test, LD50, i.e., lethal dose 50 or median lethal dose value, was calculated. LD50 is a term used in toxicology as a measurement of a lethal dose of a substance. Specially, the LD50 represents the dose at which a substance is lethal for 50% of tested subjects. The test was carried out as per Organisation for Economic Co-operation and Development Guidelines 425 (OECD) guidelines. For the experiment, six different test groups were prepared containing six female mice in each group and increasing doses of MFE were administered intraperitoneally ranging from 400 to 2000 mg/kg dose following the limit test procedure. Mice in all the groups were monitored until 24 h. LD50 value was determined by a method described by Ghosh (2007). The study was approved, and Institutional Ethics Committee (IEC) guidelines were followed during the conduct of experiments.

$LD50 = LD100$

– (Product a × b/ number of animals in each group)

where,

a = Dose difference (Dose in given interval – Dose in upper interval)

b = Mean mortality (No. of dead mice in given interval + No. of dead mice in upper interval)/ 2

## Preparation of diabetic mice

Diabetes was induced by administering 150 mg/kg dose of alloxan intraperitoneally, prepared in ice-cold citrate buffer (0.1 M, pH 4.5). After injection, mice had free access to food and water. After 48 h of alloxan injection, blood glucose in fasting state was checked (measured using gluco-stix, SD Check Gold, Korea) and the levels  $\geq 200$  mg/dL were considered high blood glucose in mice (Syiem et al. 2002).

## Experimental design

For all the experiments, albino mice were divided into four groups comprising six mice in each group:

Group 1: Normal control mice (NCM); administered only with distilled water.

Group 2: Diabetic control mice (DCM); administered only with distilled water.

Group 3: Diabetic mice; administered 50 mg/kg dose of ascorbic acid (D + AA).

Group 4: Diabetic mice; administered 250 mg/kg dose of MFE (D + MFE).

Doses were administered to mice on every alternate day intraperitoneally for 28 days, according to the mentioned method by Bora et al. (2018a, b). In the end of the treatment, mice were killed by cervical dislocation and dissection of the mice was for further detailed analysis. Liver and kidneys from each individual group were removed for the subsequent studies.

## Tissue homogenization

An extraction buffer (10% w/v) containing 10 mM HEPES (pH 7.4), 0.2 M mannitol, 50 mM sucrose, and 1 mM EDTA was used on half a section of liver and kidney tissues and was homogenized. This extract was used to separate the mitochondrial and cytosolic fractions from the section of the tissues used according to the procedure given by Graham (2002) with some modifications in the procedure as delineated earlier (Patar et al. 2017). Tissue homogenate was centrifuged at 1000 g for about 10 min at a controlled temperature of 4 °C for sedimentation of the cell nuclei and the cellular debris. The supernatant was accumulated and centrifugation was done at 750 g for a possible of 10 min and at a regulated temperature of 4 °C. To acquire the mitochondrial fraction, the resulting mitochondrial pellet was washed continuously by suspending mildly in an extraction buffer and then again re-sedimented at 7500 g for 10 min at a temperature of 4 °C. Now, the post-mitochondrial supernatant obtained after washing was centrifuged further at 15,000 g for 10 min at a maintained temperature of 4 °C and the final supernatant obtained was used as cytosolic fraction. An extraction buffer (10% w/v) of 25 mM HEPES (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.1% (w/v) of CHAPS, and 10 µg/mL of aprotinin was used to homogenize a small section of the remaining tissue according to the procedure described by Meki et al. (2004). Successively, at 13,000 g, the tissue homogenate was centrifuged at 4 °C for 15 min and the western blot technique was used for estimation of the resulting supernatant fraction.

## Quantification of protein

The protein concentrations in tissue homogenates were estimated by Bradford method (1976) using BSA as standard.

## Lipid peroxidation assay

TBA receptive substances (TBARS) were measured following the protocol of Ohkawa et al. (1979) for lipid peroxidation assay. The method implemented is as follows: 1.5 mL of glacial acetic acid (pH 3.5) with a concentration of 20%

was added along with 0.2 mL of 8.1% SDS, 1.5 mL of 0.8% TBA, 0.7 mL of distilled water, and 0.1 mL of sample or TEP as standard pipetted into test tubes. The tubes were vortexed and kept in a hot water bath at 95 °C for 1 h. To make the last volume to 5 mL, 0.7 mL of distilled water was added to every test tube. At that point, a 5-mL mixture of butanol and pyridine in 15:1 ratio respectively was added, and tubes were shaken vigorously for 2 min. The tubes were centrifuged at 3000 rpm for 10 min at 25 °C. The supernatant was collected, and the absorbance was estimated at  $\lambda$ 532 nm utilizing the UV–visible spectrophotometer (Cary 50, Varian Bio) against a reference blank. The outcome was communicated as nmol malondialdehyde (MDA)/mg protein.

### Estimation of protein carbonyl

The degree of protein carbonyl was estimated using the process of Levine et al. (1990) with certain adjustments. In the process, the homogenized tissues were incubated with streptomycin sulfate (10% w/v) and centrifuged at 11,000 g for 10 min at 4 °C. Similarly, the supernatant was equally partitioned into two test tubes: one tube with 10 mL 2,4-dinitrophenylhydrazine (DNPH) and the other with 2 M HCl to serve the purpose of reference blank. The test tubes were left to react at room temperature in complete darkness for 1 h, with occasional mixing every 15 min. Proteins were then precipitated with the addition of 20% trichloroacetic acid. For the disposal of supernatant, the tubes were again centrifuged at 11,000 g for 10 min at 4 °C followed by the washing of rest of the protein pellet cautiously with 1 mL ethanol:ethyl acetic acid solution (1:1 v/v) and centrifuged at 10,000 g for 10 min at 4 °C. The resulting pellet was solubilized in 6 M guanidine hydrochloride. Utilizing the UV–visible spectrophotometer, spectra were collected which allowed assessing the protein concentration from absorbance recorded at  $\lambda$ 360 nm. The standard molar extinction coefficient of aliphatic hydrazine is  $22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  and was used to measure the level of protein carbonyl. The outcome was communicated as nmol protein carbonyl/mg protein.

### Ultrastructural study

The technique of Hayat (2012), with certain modifications, was utilized to prepare tissues for ultrastructural study. Furthermore, the liver and kidney tissues were collected and minced to smaller pieces and transferred into Karnovsky's fixative. Then, 0.1 M cacodylate buffer was used to wash the fixed tissue pieces. Then, the tissue pieces were centrifuged at 10,000 g for 1 min. The pieces of tissue were fixed by immersing in osmium tetra oxide (1%) along with dehydrating the tissues in acetone (ascending grades 70–95%). Then, the pieces were inserted in a blend of embedding medium

and propylene oxide followed by segmenting with an ultramicrotome. The tissue segments were finally stained with uranyl acetate reagent and viewed under a transmission electron microscope (JEM-2100, JEOL).

### Western blot analysis

This analysis was carried out by first preparing a mixture of the sample that will be analyzed, and mixed with  $5 \times$  Laemmli used as a buffer in the 4:1 ratio and incubating this mixture in a hot water bath for about 5 min. Samples now collected were loaded onto wells and resolved on a 12% SDS–polyacrylamide gel. This sample after resolving underwent electrophoresis and the proteins separated out in the electrophoresis gel was transferred to nitrocellulose membrane with the help of Bio-Rad Mini Trans-Blot electrophoretic unit maintained at a constant voltage of 100 V for about 1 h. The membrane now was called the electroblotted membrane and this membrane was next transferred to a blocking solution that contains 5% of skim milk which was added to tris-buffered saline (TBS) to prepare this solution; this setup was maintained for about 3 h at room temperature. After being washed thoroughly with TBS followed by Tween tris-buffered saline (TTBS), the resulting membrane was now incubated with substances like anti-caspase 3 (1:500), anti-caspase 9 (1  $\mu\text{g}/\text{mL}$ ), and anti-Bcl-2 (1:1000) or anti- $\beta$ -actin (1:1000) antibodies and was to be maintained overnight at a temperature of 4 °C. Antibody  $\beta$ -actin was used in the experiment as a loading controlling agent. Now, the membrane was again washed with the same chemical TBS and TTBS, but this time incubated in a solution of hydrogen peroxidase (HRP) in conjugation with secondary antibodies in the ratio (1:5000) for about 2 h. At room temperature, the addition of the following substrate (TMB/H<sub>2</sub>O<sub>2</sub>) was done to spot for any color development. The reaction was concluded by washing the membrane with double-distilled H<sub>2</sub>O and the photograph of the resulting membrane was taken using an HP Scanjet 7400C. The blots underwent quantification densitometrically using the software My Image Analysis, a product of Thermo Fisher Scientific Inc. The results were expressed as the relative intensity comparing it with the  $\beta$ -actin protein.

### TUNEL assay

For the in situ detection of DNA fragments in a given sample, fluorescein-based TUNEL assay was the method adopted to perform the detection. In this method, 5- $\mu\text{m}$ -sized sections of paraffin-embedded tissue were prepared and arranged sequentially, where each section was adhered to albumin and glycerol in the ratio 1:1. The prepared coated slides were then allowed to dry at room temperature. These sections were collected and de-paraffinized followed by rehydration

and washing with the solution of phosphate-buffered saline (PBS) and then incubated along with proteinase K which was prepared by mixing 20 µg/mL in 10 mM Tris–HCL as the buffer, pH 7.5, which was then kept for about 15 min at room temperature. Sections were then collected again and washed with PBS. The sections now obtained were subsequently incubated in a TUNEL reaction mixture comprising TdT enzyme and the fluorescein-labeled dUTP nucleotides maintained in the dark for about 1 h and the temperature was maintained at 37 °C; these sections were now followed by counterstaining with DAPI. After counterstaining, these sections were finally covered with a coverslip and then viewed under a confocal microscope (TCS SP5, Leica, Germany).

## Statistical analysis

Statistical analysis was achieved by one-way ANOVA supported by Tukey's post hoc test to assess notable differences between experimental groups using the statistical package "IBM SPSS Statistics 19.0" for Windows OS. Results are articulated as mean ± SEM for five mice in each experimental group. Statistical significance was established at  $p < 0.05$ .

## Results

### Qualitative phytochemical analysis

Flavonoids, tannins, glycosides, and terpenes were strongly present (++) in the MFE, whereas alkaloids and saponins were slightly present (+) as shown in Table 1.

### Toxicological evaluation

The smaller the LD50 value, the more toxic would be the chemical. As per observations and calculations

**Table 1** Phytochemical screening results of *Phlogacanthus thyrsoiflorus* flower extract fractions

Metabolites	Methanolic flower extract (MFE)
Alkaloids	+
Tannins	++
Glycosides	++
Flavonoids	++
Terpenes	++
Saponins	+

++ highly present, + low, - absent

**Table 2** Toxicological study of different doses of MFE of *Phlogacanthus thyrsoiflorus*

Groups	Dose (mg/kg)	Mortality (x/n) Toxicity in mice
Group 1	400	0/6
Group 2	800	0/6
Group 3	1200	0/6
Group 4	1600	0/6
Group 5	2000	0/6

*n* number of mice in each group

(Table 2), the LD50 value of MFE was found out to be more than 2000 mg/kg dose, which implies that even in the higher dose the extract is safe and not toxic to mice.

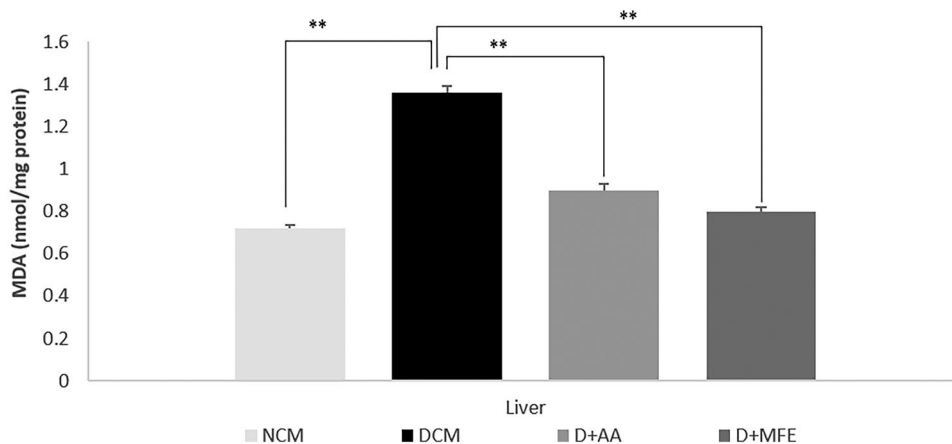
### MFE inhibits lipid peroxidation

Lipid peroxidation was confirmed in liver and kidney tissues of alloxan-induced mice where MDA level increases notably by 74% ( $1.27 \pm 0.08$ ) in liver and 80% ( $0.72 \pm 0.03$ ) in kidney compared to the normal control group (liver  $0.73 \pm 0.03$ , and kidney  $0.40 \pm 0.008$ ). In contrast to the diabetic control group, ascorbic acid-treated group exhibited a significant decrease of 35% ( $0.83 \pm 0.09$ ) in liver and 18% ( $0.59 \pm 0.03$ ) in kidney respectively in MDA level. Similarly, the same decreasing pattern in the MDA level was determined in liver, and kidney tissues of MFE-treated group showed a significant difference of 31% ( $0.87 \pm 0.04$ ) and 18% ( $0.59 \pm 0.03$ ) respectively against the diabetic control group in the study. This study suggests that inhibition of lipid peroxidation is carried out in treatment groups as showed in Figs. 1 and 2.

### MFE inhibits protein carbonylation

In Figs. 3 and 4, protein carbonyl content, which is a measure of oxidative damage done to the proteins, showed a decrease in the level which was similar to the level of decrease shown when treated with ascorbic acid in alloxan-induced mice. As compared to the DCM, protein carbonyl level in liver was decreased by 10% ( $6.42 \pm 0.24$ ) and 11% ( $6.33 \pm 0.19$ ) in MFE and ascorbic acid treatment, respectively. Similarly, protein carbonyl level in kidney was decreased by 14% ( $5.66 \pm 0.07$ ) and 17% ( $5.45 \pm 0.07$ ) in MFE and in ascorbic acid treatment, respectively. In alloxan-induced diabetic mice, protein molecules were highly oxidized as protein carbonyl content was found to be 41% and 68% higher than that in normal mice (liver,  $7.14 \pm 0.15$  vs.  $5.06 \pm 0.05$ ; kidney,  $6.60 \pm 0.14$  vs.  $3.92 \pm 0.15$ ).

**Fig. 1** Effect of MFE on lipid peroxidation in liver of alloxan-induced diabetic mice. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  against the diabetic control group



### MFE ameliorates ultrastructural injuries

An ultrastructural study was performed to examine the effect of MFE on oxidative stress-mediated cellular modifications in liver and kidney of selected experimental groups. Results of the study are shown:

**Liver** Studies suggested that prototypical ultrastructures of hepatocyte containing round nucleus exhibit dispersed chromatin and prominent nucleoli in the normal control mice as opposed to deformed nucleus comprising almost dissolved chromatin and nucleus in the diabetic control mice. However, from the subtly visible nuclei and dispersed chromatin present within the round nucleus, it was evident that MFE-treated mice displayed an indistinguishable effect as ascorbic acid on restoring hepatic alterations to relatively normal, as illustrated in Fig. 5.

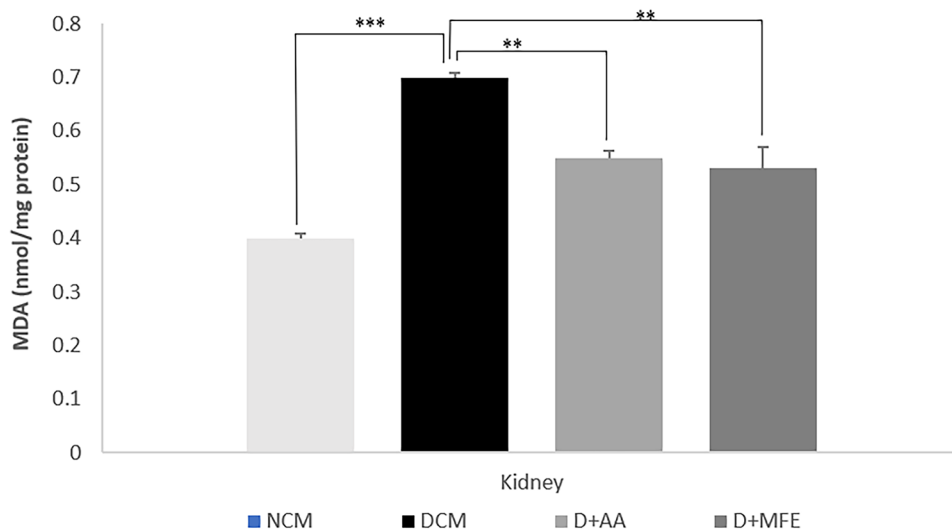
**Kidney** As showed in electron micrographs, mitochondria in the renal proximal tubule of normal mice maintained

their structural integrity with dense matrix were compact with thin and uniform cristae, and were surrounded by clear membrane, whereas in diabetic kidney numerous dysmorphic mitochondria were seen in renal proximal tubular cells, including swelling and disrupted cristae architectures. Some mitochondria exhibited dissolved membranes and complete loss of cristae. Moreover, the smooth endoplasmic reticulum (SER) got deformed, abnormally elongated, disrupted, and dissolved. However, MFE treatment was able to restore these changes up to the similar ultrastructural features shown by the ascorbic acid such as few swollen, disrupted mitochondria and most mitochondria exhibited less damaged cristae structure, as described in Fig. 6.

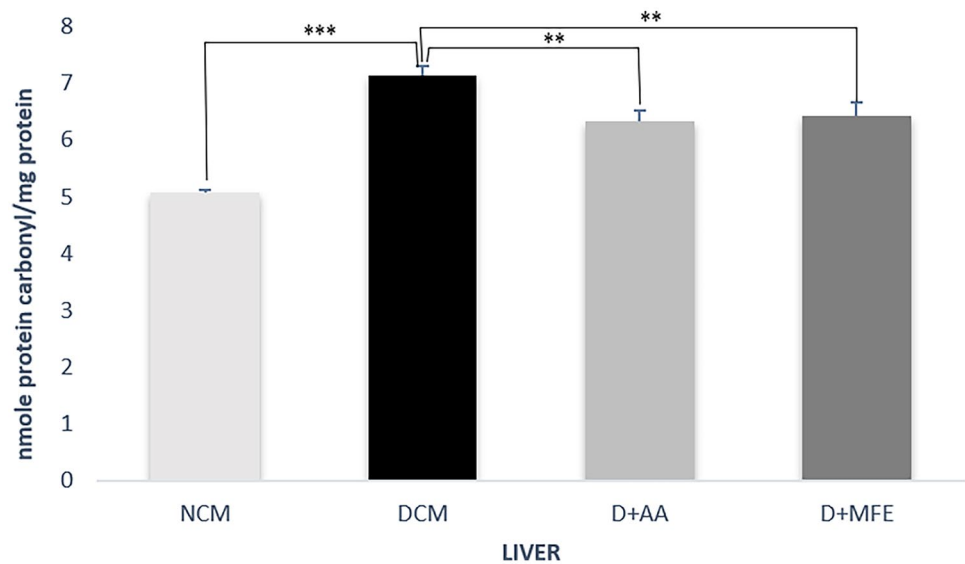
### MFE modulates apoptosis

Western blot technique was done for the analysis of expressions of proteins—caspase 3 expression was upregulated in the case of diabetic control mice along with significant increases of 2.69-fold ( $0.48 \pm 0.03$  liver)

**Fig. 2** Effect of MFE on lipid peroxidation in kidney of alloxan-induced diabetic mice. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  against the diabetic control group



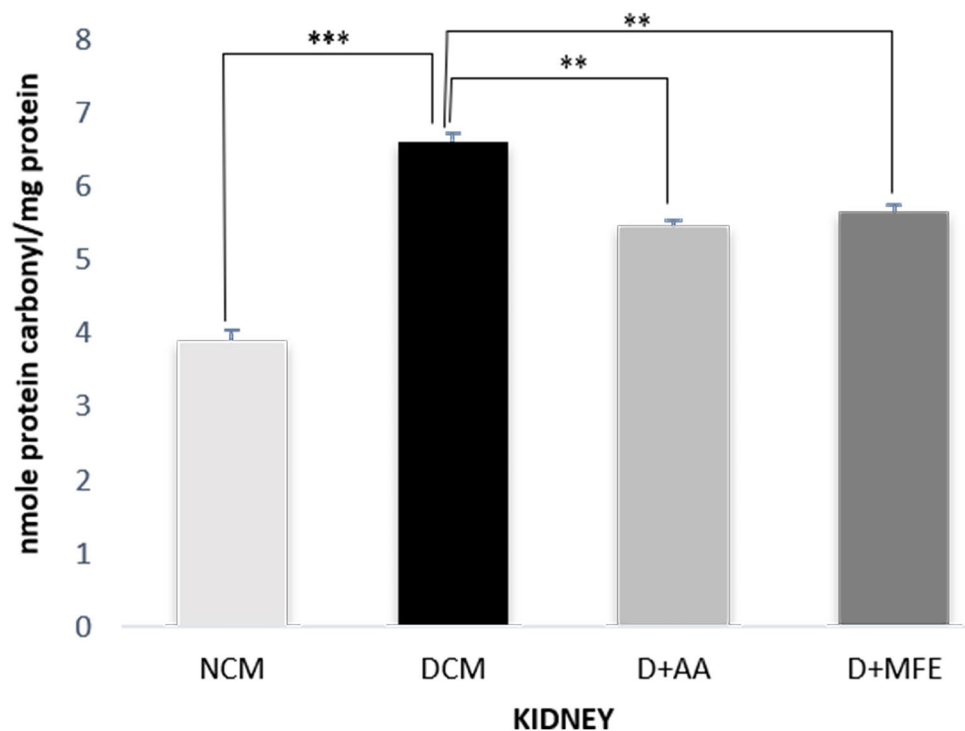
**Fig. 3** Effect of MFE on protein carbonylation in liver of alloxan-induced diabetic mice. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  against the diabetic control group



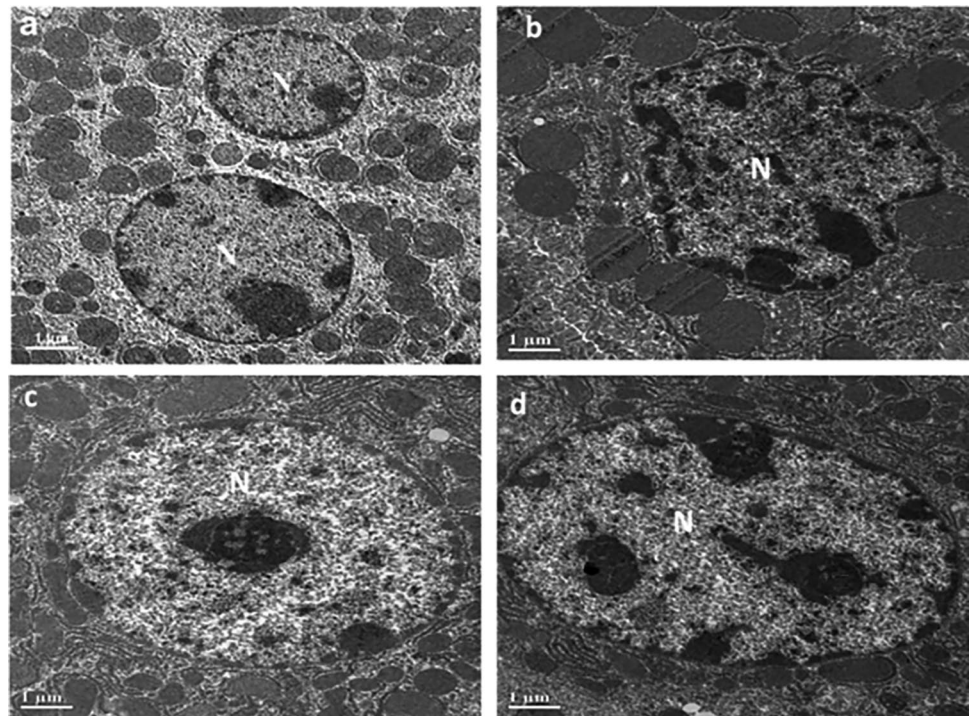
and 4.33-fold ( $0.48 \pm 0.01$  kidney) in protein level as compared to the normal control mice ( $0.13 \pm 0.01$  and  $0.09 \pm 0.006$ ), whereas in the ascorbic acid-treated mice, the expression of caspase 3 is noticeably decreased by 0.54-fold ( $0.22 \pm 0.006$  liver) and 0.60-fold ( $0.19 \pm 0.005$  kidney) in protein level as compared to the diabetic control mice. Similarly, in the MFE-treated mice, caspase 3 expression was downregulated with a significant decrease of 0.58-fold ( $0.2 \pm 0.003$  liver) and 0.52-fold ( $0.23 \pm 0.008$  kidney) in protein level against diabetic control mice (Figs. 7 and 8).

On the contrary, it has been observed that anti-apoptotic Bcl-2 expression was downregulated with a significant decrease of 0.69-fold ( $0.12 \pm 0.01$  liver) and 0.42-fold ( $0.19 \pm 0.006$  kidney) in protein level in diabetic control mice compared to normal control mice ( $0.39 \pm 0.02$  and  $0.33 \pm 0.009$ ), whereas Bcl-2 expression was significantly increased by 1.25-fold ( $0.27 \pm 0.01$  liver) and 0.26-fold ( $0.24 \pm 0.01$  kidney) in ascorbic acid-treated mice compared to the diabetic control mice. Similarly, in the MFE-treated, Bcl-2 expression was altered positively as evident from 0.75-fold ( $0.21 \pm 0.008$  liver) and 0.21-fold ( $0.23 \pm 0.005$  kidney)

**Fig. 4** Effect of MFE on protein carbonylation in kidney of alloxan-induced diabetic mice. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  against the diabetic control group



**Fig. 5** Transmission electron micrographs showing ultrastructural changes in hepatocytes of the experimental groups containing normal control mice **a**, diabetic control mice **b**, diabetic mice treated with the ascorbic acid **c**, and methanolic flower extract **d**. *N*, nucleus



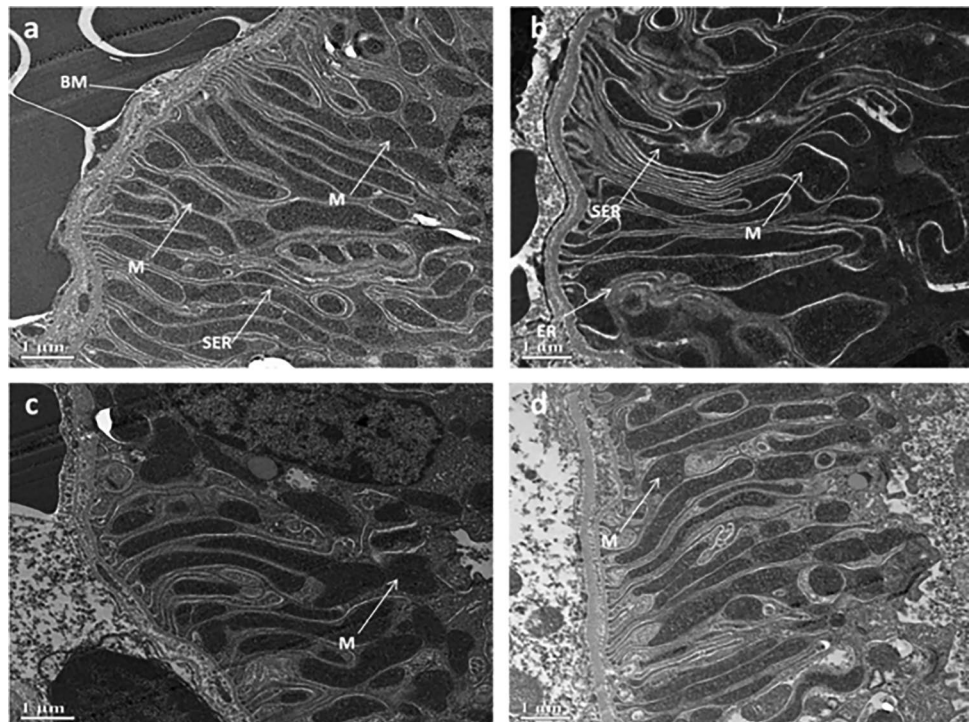
increases in protein level against the diabetic control in the study (Figs. 9 and 10).

#### TUNEL assay

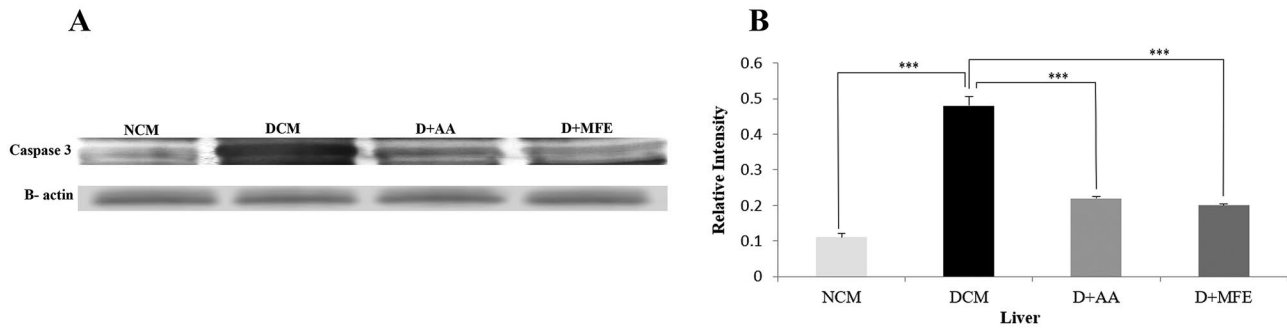
TUNEL assay studies were used for the analysis of apoptotic cell death as depicted in Figs. 11 and 12.

**Liver** Figure 12 shows the results in liver tissue. In comparison to the normal control, percentage of TUNEL-positive cells was measured to be 75.7% in diabetic control mice. However, 34.2% and 57.1% of TUNEL-positive cells were measured in both ascorbic acid- and MFE-treated mice indicating significant reductions in level of apoptotic cells death against diabetic control in the study.

**Fig. 6** Transmission electron micrographs showing ultrastructural changes in renal proximal tubule of the experimental groups containing normal control mice **a**, diabetic control mice **b**, diabetic mice treated with the ascorbic acid **c**, and methanolic flower extract **d**. *M* mitochondria, *SER* smooth endoplasmic reticulum, *BM* basement membrane







**Fig. 7** Effect of MFE on caspase 3 expression in liver of alloxan-induced diabetic mice. Western blot analysis **A** the corresponding relative intensity of the western blot of Caspase 3 after normaliza-

tion with  $\beta$ -actin protein **B**. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \*\* $p < 0.01$  and \*\*\* $p < 0.001$  against the diabetic control (DCM) group

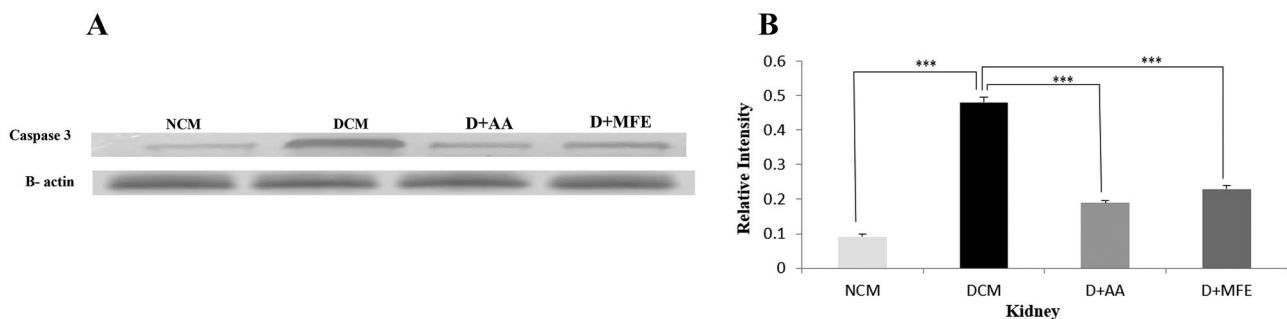
**Kidney** Figure 11 shows the result in kidney tissues. It was evaluated from the confocal microscopic images that in diabetic control mice, 74.2% of cells were measured to be TUNEL-positive cells when compared to the normal control mice, whereas significant reductions by 48.5% in ascorbic acid treated and 48.5% in MFE treated in level of apoptotic cell death were measured than those in the diabetic control in the study.

## Discussion

Alloxan, which is also assigned as alloxan monohydrate, with the formula  $OC(N(H)CO)2C(OH)2$ , is a toxic counterpart of glucose that demolishes  $\beta$ -cells (insulin-producing cells) of pancreas when directed to mice or other animal species as it favorably assembles in  $\beta$ -cells through uptake via the GLUT2 glucose transporter and causes insulin-dependent diabetes mellitus (called “alloxan diabetes”) in these animals, alike to type 1 diabetes in humans (Lenzen 2008). Practically, in the presence of intracellular thiols, alloxan initiates ROS production in a cyclic reaction with its depletion product, dialuric acid. Free radicals

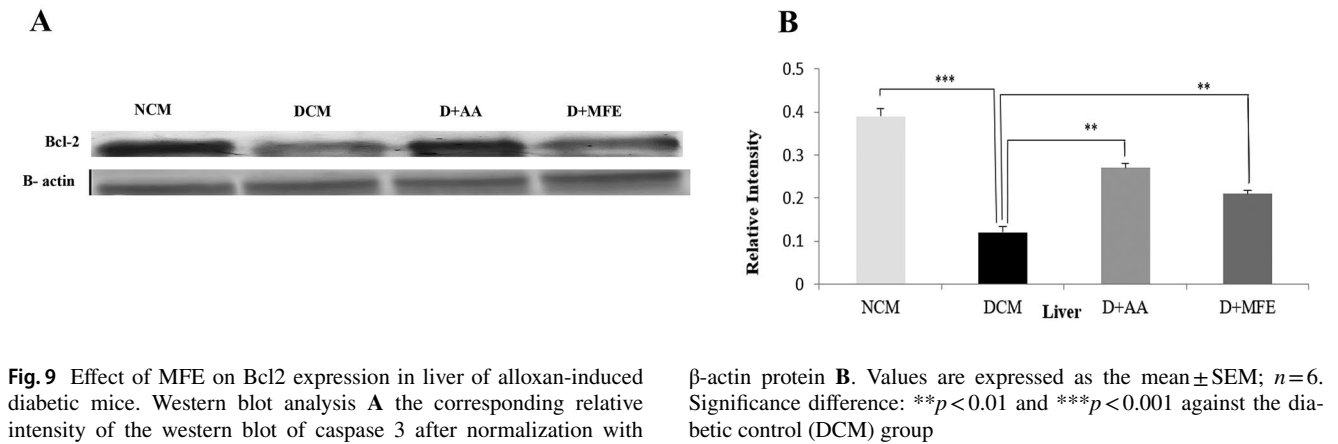
formed during the redox reaction activate the toxic action of alloxan in beta cells (Danilova et al. 2015). Validating with the reports, our study also illustrated consequent advancement of oxidant in alloxan-induced diabetic mice. It is demonstrated in our study that MFE could diminish alloxan-triggered oxidant generation in diabetic mice, and this is in line with other findings that manifested searching of oxidant by MFE in various experimental systems (Kumar et al. 2001).

Biomolecular damage by ROS makes various byproducts, which possess characteristics of biomarker for oxidative stress. For example, an essential biomarker is MDA, which is an aldehyde molecule generated in polyunsaturated fatty acid peroxidation (Draper and Hadley 1990). Various other eminent biomarkers such as protein carbonyls form when there is a direct oxidation by ROS in certain amino acid residues or by their interconnection with aldehyde containing lipid peroxidation products (Dalle-Donne et al. 2003). Under the hyperglycemic state, the advancement of these biomarkers was described from various studies conducted on diabetic patients and animal models (Pan et al. 2010; Saio et al. 2012). According to our study, a subsequent increase of MDA along with protein carbonyl levels was



**Fig. 8** Effect of MFE on caspase 3 expression in kidney of alloxan-induced diabetic mice. Western blot analysis **A** the corresponding relative intensity of the western blot of caspase 3 after normaliza-

tion with  $\beta$ -actin protein **B**. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \*\* $p < 0.01$  and \*\*\* $p < 0.001$  against the diabetic control (DCM) group



**Fig. 9** Effect of MFE on Bcl2 expression in liver of alloxan-induced diabetic mice. Western blot analysis **A** the corresponding relative intensity of the western blot of caspase 3 after normalization with

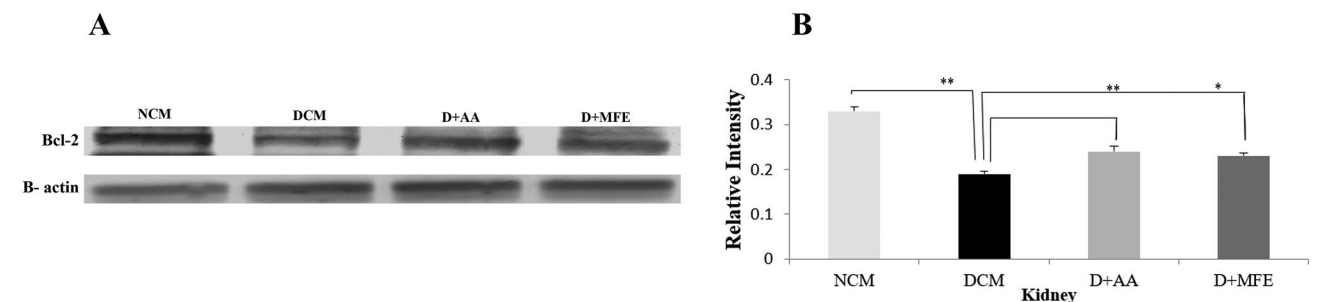
$\beta$ -actin protein **B**. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \*\* $p < 0.01$  and \*\*\* $p < 0.001$  against the diabetic control (DCM) group

also observed in alloxan-induced diabetic mice. Repression of lipid peroxidation and protein carbonylation by MFE was depicted as the result of this study. Another feature of lipid peroxidation products is that they may persuade genetic expression of fibrogenic cytokines and increase the collagen synthesis by instigating the arousal of hepatic stellate cells (HSCs) (Parola et al. 1992; Lee et al. 1995). According to this study, fibrosis, portrayed by the unrestrained synthesis and assembling of collagen and other extracellular matrix proteins divulged tissue injuries, was marked in case of alloxan-persuaded diabetic mice. In diabetic mice, the ultrastructural advancement also showed severe hepatic and nephron cell abnormalities as compared to that in the normal mice. However, like ascorbic acid, the capability of MFE to diminish tissue destruction can be characterized to its free radical chain reaction termination capacity for inhibition of lipid peroxidation, in directing an antioxidative property.

In the hyperglycemic state of diabetes, the operation of cell death by apoptosis is a comparatively omnipresent occurrence in a diversity of cell types, including hepatocytes and nephrocytes. The procedures balancing these processes are composite and incompletely understood. However, the mitochondrial-mediated apoptotic track, activated by disturbance of membrane potential, has been described in

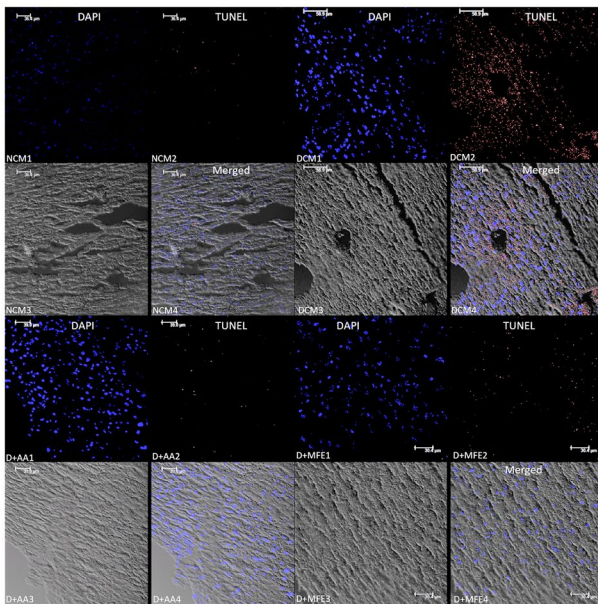
many cases of hyperglycemia-induced oxidative stress (Cai et al. 2002; Rashid et al. 2012). This is modulated normally by Bcl-2 and another of its cousin antiapoptotic proteins by means of blocking the conversion outlet development through interrelating with proapoptotic Bcl-2 family proteins that ultimately hampers them from forming homodimers. Whether it is by mitochondrial or non-mitochondrial apoptotic pathway is dependent finally on arousal of effector caspases such as caspase 3. Cells inferior in caspase 3 were observed to be resistant to apoptosis (Yang et al. 2001). Caspase 3 arousal and apoptosis persuaded in high levels of glucose were found to be vanquished by caspase 3-specific inhibitor, indicating that activation of caspase 3 is involved in the hyperglycemia-induced apoptotic cell death (Cai et al. 2002).

In various tissues like in myocardium and in hepatic, regulation of Bcl-2 family protein expression, leakage of cytochrome c and caspase 3 activation enzymes, and cell death via apoptosis are quite perceptible in alloxan-induced diabetic animal models (Cai et al. 2002; Francés et al. 2010). Confirming with the reports, our research study has also illustrated the upregulation of caspase 3, whereas downregulation of expression of Bcl-2 in alloxan-induced diabetic mice suggests the mitochondrial-mediated activation

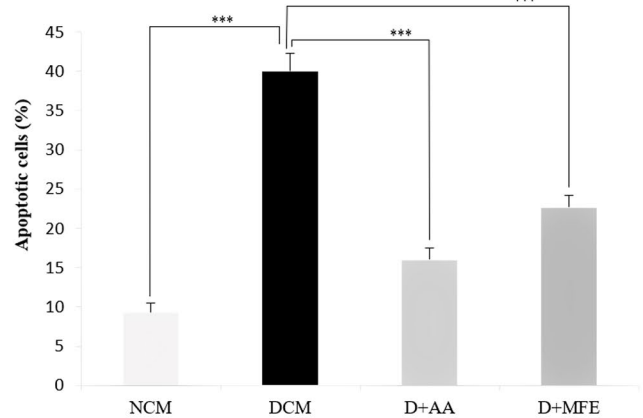


**Fig. 10** Effect of MFE on Bcl2 expression in kidney of alloxan-induced diabetic mice. Western blot analysis **A** the corresponding relative intensity of the western blot of Caspase 3 after normaliza-

tion with  $\beta$ -actin protein **B**. Values are expressed as the mean  $\pm$  SEM;  $n=6$ . Significance difference: \*\* $p < 0.01$  and \*\*\* $p < 0.001$  against the diabetic control (DCM) group



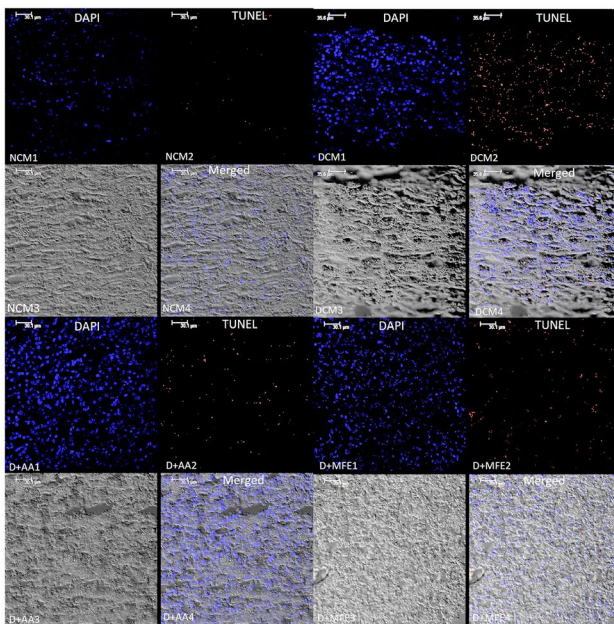
**Fig. 11** The result shows DAPI staining, TUNEL staining, and merge (DAPI+TUNEL) confocal microscopic images (left) and percentage of apoptotic cells (right) in liver tissue of normal control mice (NCM), diabetic control mice (DCM), diabetic mice treated with the



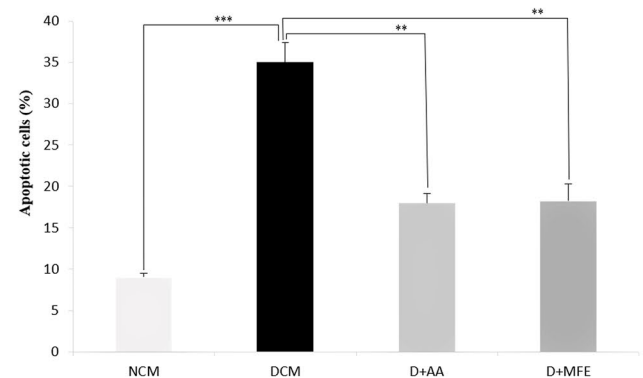
ascorbic acid (D+AA), and MFE (D+MFE). Values are expressed as the mean  $\pm$  SEM. Significance difference: \* $p < 0.05$  and \*\* $p < 0.01$  against the diabetic control group

of apoptosis. Regulation of apoptosis by MFE is quite perceptible from various studies remarkably (Sharma et al. 2007; Thiagarajan et al. 2012); however, the means of MFE actions could be cell death or cell survival relying

on the pathophysiological conditions. According to our study, a cell survival means of action as the ascorbic acid against apoptosis persuaded by hyperglycemia was shown by MFE. The downregulated caspase 3 whereas upregulated



**Fig. 12** The result shows DAPI staining, TUNEL staining, and merge (DAPI+TUNEL) confocal microscopic images (left) and percentage of apoptotic cells (right) in kidney tissue of normal control mice (NCM), diabetic control mice (DCM), diabetic mice treated with the



ascorbic acid (D+AA), and MFE (D+MFE). Values are expressed as the mean  $\pm$  SEM. Significance difference: \* $p < 0.05$  and \*\* $p < 0.01$  against the diabetic control group

expressions of Bcl-2 were considered in this case, suggesting a positive role of MFE via reinstating the potential of mitochondrial membrane against hepatocyte and nephrocyte apoptotic death in alloxan-induced diabetic mice. However, in this study, it is not known about the operation by which MFE regulates the expression of selected apoptotic marker proteins.

Another hallmark of apoptosis is DNA fragmentation which is a result of hyperglycemia-induced overproduction of ROS. According to our research study, fragmentation of DNA is ceaselessly perceptible from numbers of TUNEL-positive cells recognized in alloxan-induced diabetic mice as compared with the normal mice. In dissimilitude, fewer TUNEL-positive cells were recognized in MFE treatment as the ascorbic acid, inferring protective effect of MFE against ROS-induced fragmentation in DNA as it is further perpetuated by the affirmation that correlates to the protective effect of MFE against radiation-induced plasmid pBR322 DNA damage (Kumar et al. 1999).

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