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Original Research

Therapeutic Potential of *Dillenia indica* L. in Attenuating Hyperglycemia-Induced Oxidative Stress and Apoptosis in Alloxan-Administered Diabetic Mice

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Abstract

Background: Hyperglycemia-induced oxidative stress accelerates the process of apoptosis in tissues. *Dilleniaindica* (DI) is a medicinal plant, and its fruit contains many therapeutic properties. The therapeutic activity of the Methanolic Fruit Extract (MFE) of DI in attenuating oxidative stress and apoptosis in the liver and kidney tissues of alloxan-induced diabetic mice was analyzed in the present study. **Methods**: High-Performance Thin Layer Chromatography (HPTLC) profiling of MFE was conducted. GLUT4 protein expression analysis and lipid peroxidation assays were conducted to check for MFE effect by administering in diabetic mice. An ultrastructural study was conducted for both the tissues. In apoptotic studies, the TUNEL assay and apoptotic protein expression analysis was conducted. **Results**: High-Performance Thin Layer Chromatography (HPTLC) profiling of MFE showed the presence of two crucial antioxidants, ascorbic acid, and naringenin. In GLUT-4 protein expression analysis, MFE suppresses hyperglycemia by upregulating GLUT4 protein expression. Lipid peroxidation assay showed a decrease in malondialdehyde (MDA) upon MFE administration in diabetic mice. An ultrastructural study was conducted, and MFE was found to restore cellular alterations in diabetic tissues. In apoptotic studies, the TUNEL assay shows that MFE treatment showed fewer apoptotic cells than the diabetic group. The study also observed decreased caspase 3 protein expression and increased Bcl-2 protein expression. **Conclusions**: Therefore, it is inferred from the study that MFE can exert a protective effect by suppressing hyperglycemia and modulating oxidative stress and apoptosis in alloxan-administered diabetic mice.

Keywords: *Dillenia indica* extract; HPTLC; alloxan; hyperglycemia; oxidative stress; apoptosis

1. Introduction

Diabetes mellitus (DM) is a group of metabolic alterations characterized by hyperglycemia resulting from defects in insulin secretion, action, or both [1]. Glucose transporter 4 (GLUT4) is a prime carrier for glucose transport which transports blood glucose into muscle cells and fat tissues. Disruption in the GLUT4 expression induces insulin resistance [2]. Therefore, it has been considered a therapeutic target for pharmacological intervention strategies to control diabetic hyperglycemia [3]. However, in diabetic conditions, the intracellular Reactive Oxygen Species (ROS) level is subsequently increased by the mitochondrial respiratory chain reaction and other activities. The processes promoting ROS production include glucose autoxidation, glycosylation, glucosamine, and polyol pathways [4,5]. A combination of ROS overproduction and concomitant downregulation of the activity of antioxidant enzymes leads to the alteration in the redox homeostasis, causing oxidative stress in diabetes [6,7]. ROS attack causes membrane disintegration and releases cytochrome C into the cytosol, triggering apoptosis [8].

Apoptosis, commonly called programmed cell death, is a natural process of cell death [9]. The protease enzymes known as caspases can be categorized as initiator caspases

(caspase-9) and effector caspases (Caspase-3, caspase-6, and Caspase-7), and they are essential apoptosis-related enzymes [10]. Cytochrome-*c* binds to the cytosolic Apaf-1 (apoptosis protease activating factor-1) and triggers the formation of an apoptosome complex. This complex recruits initiator pro-caspase-9 to its Caspase recruitment domain, allowing auto-activation and then proteolysis [11]. The process, in turn, activates downstream executor caspases-3, 6, and 7 for cleavage of cellular substrates leading to apoptotic cell death [12,13]. Another protein family known as B-cell lymphoma-2 (Bcl-2) is seen to control apoptosis through a mechanism that controls the permeability of the mitochondrial membrane. Bcl-2 proteins are classified as either pro- or anti-apoptotic, depending on whether they inhibit or accelerate the apoptotic process [14,15].

Although several therapies are available for treating diabetes, like expensive medications, there are various limitations linked to it [16]. Medicinal plants with potent pharmaceutical properties could be potent sources of treatments for the existing remedies [17,18]. DI is one such medicinal plant chosen for the study since it has many therapeutic properties. DI, commonly recognized as Elephant apple or Indian catmon (English), is widely spread in India, Bangladesh, Nepal, and Sri Lanka [19]. It was also found traditionally that in various parts of northeast India, the juices of bark and leaves were mixed and given orally to treat cancer and diarrhea [20,21]. Also, different literature review on the plant shows that the leaves, bark, and fruits of DI have extensive medicinal values, including antioxidant potential [22]. Oral synthetic hypoglycemic drugs, the primary source for controlling diabetes, fail to reverse the course of its complications, and turn, worsen as they demonstrate side effects. Natural products could be promising lead candidates for significant drug development with benefits such as availability ease, low cost, and most minor side effects, making them the key players of available therapies, especially in rural areas. Moreover, many bioactive chemicals found in plants are free from undesirable side effects and possess decisive pharmacological actions [23]. The present experimental study was intended to contribute to particular research significance so that natural products could be more opted in combating diabetes and its related complications, preventing significant side effects in the body bought about by synthetic drugs. Hence, a recent study was conducted; to examine the potential impact of DI fruits on hyperglycemia and oxidative stress-related apoptosis in the kidney and liver tissues of diabetic mice.

2. Materials and Methods

2.1 Chemicals

Metformin and alloxan were obtained from Sigma-Aldrich co. USA. Sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), and H_20_2 were procured from Merck life sciences Pvt. Ltd. India. Analytical grade Chemicals were obtained from Merck Co. (Mumbai, India), Sisco Research laboratory, Himedia, and S.D. Fine chemical Ltd. India. TUNEL assay kit was purchased from Roche, Switzerland. Anti-caspase 3 antibody was obtained from Sigma –Aldrich Co.USA, anti- Bcl2 and anti-GLUT4 antibody from Merck life sciences Pvt. Ltd. India.

2.2 Plant Material

The DI fruits were collected from Guwahati, Assam. Plant identification was made by Dr.P. B. Gurung, Curator herbarium, Botany Department, North-Eastern Hill University, Shillong, Meghalaya (Voucher No: 12056).

2.3 Experimental Animals

Healthy Swiss albino male mice (Balb/C strain) weighing 25–30 gm of body weight (b.w) were selected for the experiment. All the experiments were conducted following the Institutional Ethics Committee (IEC) guidelines, Animal model, North Eastern Hill University, Shillong, India, dated 04/12/2014. Animal models (mice) were kept in appropriate cages of polypropylene, fed standard laboratory feed, and the environment was held at a constant 25 + 2 °C temperature with a 12 hr day/night cycle.

2.4 Preparation of Extract

Harborne [24] described the method for preparing the extract. DI fruit pieces were air-dried until totally dry at room temperature. Dried fruit was grounded in powder form in a blender. It was further extracted overnight through incubation while continuously stirring with ten volumes of methanol: aqueous solution (4:1) to create Methanolic fruit extract (MFE). After the mixture was filtered, the filtrate was maintained in a rotary evaporator at 38 °C until it evaporated and dried. After that, the dried extract was kept at –20 °C until future investigations.

2.5 High-Performance Thin Layer Chromatography (HPTLC) Fingerprinting

The HPTLC fingerprinting procedure was performed using Syed *et al*. [25]. With an automatic TLC applicator Linomat-5b with N2 flow (CAMAG, Muttenz, Switzerland), aliquots of the sample (MFE)/Standards were applied on Merck, TLC silica gel 60 F 254 plates as an 8 mm broadband. After applying the sample, the plates were developed for 20 minutes in a glass tank pre-saturated with the mobile phase. White light and the Valid Diagnostics visualizer (S/N: 150503) were used to observe the developed plates. Furthermore, these plates were allowed to air dry at room temperature (R.T.) for five minutes before the chromatogram was derivatized for ten minutes using an anisaldehyde-sulfuric acid reagent previously heated to 100 °C. The derivatized plates were also observed under white light and at λ254 nm. The plates were scanned using scanner 4 (S/N: 170422) from Valid Diagnostics, India at 366 nm with win-CATS-V 1.2.3 software (CAMAG, Muttenz, Switzerland) to perform a quantitative analysis of the compounds.

2.6 Preparation of Diabetic Mice

Alloxan prepared in ice-cold citrate buffer at 150 mg/kg was given intraperitoneally to cause diabetes (0.1 M, pH 4.5) in mice. Mice had unrestricted access to food and liquids following injection. After receiving an alloxan injection for 72 hours, the mice's fasting blood glucose levels (FBGL) were measured using a glucostix (S.D. Check Gold, Korea), and mice with an FBGL of 200 mg/dL or more were diagnosed as diabetic.

2.7 Analysis of GLUT4 Protein Expression

2.7.1 Experimental Design

The study was performed in four different groups of overnight fasted mice consisting of six mice in each group:

Group 1: Normal control (NC) mice administered with only distilled water

Group 2: Diabetic control (DC) mice administered with only distilled water

Group 3: Diabetic mice administered with 50 mg/kg b.w of metformin (D+MET)

Group 4: Diabetic mice administered with 350 mg/kg b.w of MFE (D+MFE)

Doses were injected every alternate day for 21 days intraperitoneally [26]. After 21 days of treatment, the mice were killed and carefully dissected to remove the skeletal muscles of the limbs for subsequent study.

2.7.2 Skeletal Muscle Homogenization

The limb muscle was excised and extracted in 10% (w/v) extraction buffer containing sodium chloride (150 mM), 0.1% Triton X100, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate (SDS) and Tris buffer (50 mM, pH 8.0). Homogenates were centrifuged at 13000g for 15 minutes at 4 °C [27]. The supernatant was immediately collected and stored at –80 °C for GLUT4 protein expression analysis.

2.7.3 Western Blot Analysis

Western blot is an analytical technique to detect a specific protein in a sample of tissue homogenate. Sample and the 5 Laemmli buffer containing (5% SDS, 50% glycerol, 0.1% bromophenol blue, 250 mMTris-HCL, pH 6.8, and 5% of 2-mercaptoethanol) were prepared quickly. The membrane was placed in a blocking solution made of Tris-buffered saline (TBS) with 5% skim milk for 3 hours at room temperature. The membrane was treated with anti-GLUT4 antibody (0.5 g/mL) or anti-actin (1:1000) antibodies overnight at 4 °C after being washed with TBS and Tween Tris-buffered saline (TTBS). As a loading control, an actin antibody was utilized (housekeeping gene). The membrane was rewashed in TBS and TTBS and incubated with hydrogen peroxidase (HRP) conjugated secondary antibodies (1:5000) for two h at room temperature, followed by the addition of substrate (TMB/H_2O_2) for color development. The reaction was stopped by washing the membrane in double distilled water and photographed using an H.P. Scan Jet 7400C. The blots were quantified densitometrically using My Image Analysis software, Thermo Fisher Scientific Inc., and expressed as the relative intensity compared with the β-actin protein.

2.8 Study of the Effects of MFE on Oxidative Stress and Apoptosis

2.8.1 Experimental Design

The study was performed in four groups of overnight fasted mice, with three in each group.

Group 1: Normal control (NC) mice administered with only distilled water

Group 2: Diabetic control (DC) mice administered with only distilled water

Group 3: Diabetic mice administered with 50 mg/kg b.w of Ascorbic acid (D+AA)

Group 4: Diabetic mice administered with 350 mg/kg b.w of MFE (D+MFE)

Doses were administered to mice intraperitoneally by injecting every alternate day for up to 21 days [26]. At the end of the experimental period, mice were sacrificed and dissected to excise each group's liver and kidney tissues for subsequent analyses.

2.8.2 Lipid Peroxidation Assay

2.8.2.1 Preparation of Cytosolic and Mitochondrial Fractions of Tissues. According to the Graham, 2002 method, cytosolic and mitochondrial fractions of tissues were prepared by differential centrifugation. Tissues were homogenized in 10% (w/v) HEPES buffer, pH 7.4. Tissue homogenates were centrifuged at 1000 *g* for 10 minutes at four °C, and the supernatant was collected and again centrifuged at 7500 *g* for 10 minutes at 4 °C. The resulting supernatant from the second centrifugation was collected and centrifuged at 15000 *g* for 10 mins at 4 °C. The resulting supernatant obtained was the cytosolic fraction. The resulting pellet was further washed in buffer and centrifuged at 7500 *g* for 10 minutes at 4 °C to obtain the mitochondrial fraction [28,29].

2.8.2.2 Quantification of Protein. The specific enzymatic activities were determined through total protein concentration present in the fractions of the cytosolic and mitochondria through bovine serum albumin (BSA) as the standard per the given protocol [30]. The thiobarbituric acid reactive substances (TBARS) were measured through the Lipid peroxidation assay [31]. Further, MDA (malondialdehyde) levels were obtained by extrapolating the curve, and the result was expressed as nm MDA/mg protein.

2.8.3 Ultrastructural Studies

Tissues were prepared using a modified version of Hayat's [32] approach for the ultrastructural examination. Cut into small pieces, the liver and kidney tissues were

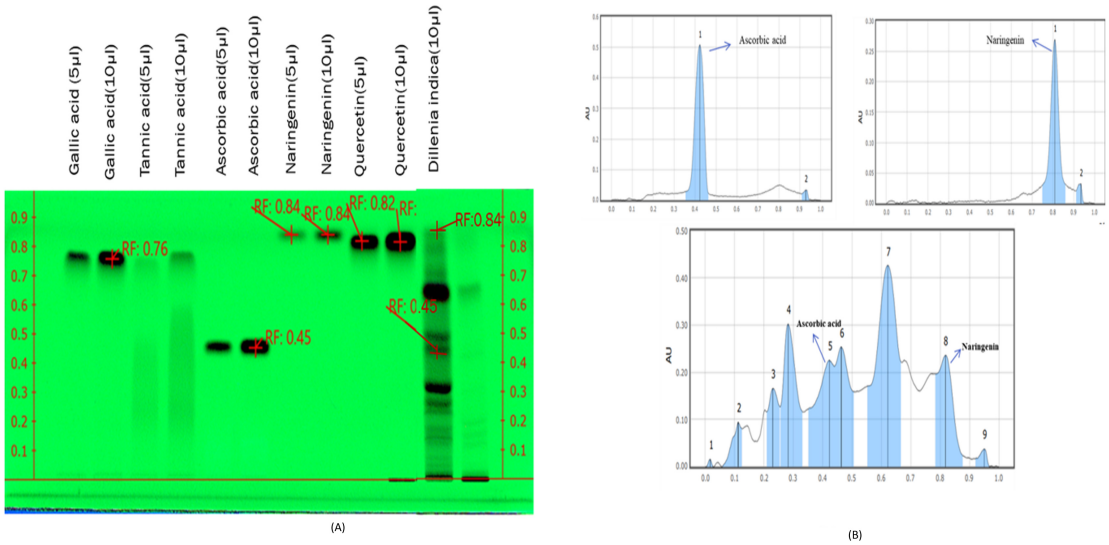

Fig. 1. HPTLC fingerprinting of Gallic acid, Tannic acid, Ascorbic acid, Naringenin, Quercetin, and MFE observed at λ254 nm. The mobile phase used is (n-butanol: glacial acetic acid: water (4:1:1 v/v/v) (A). HPTLC band chromatogram of MFE of DI compared with standards ascorbic acid and naringenin (B) HPTLC peak scanning chromatogram of MFE of DI compared with standards ascorbic acid and naringenin.

fixed in Karnovsky's fixative. The fixed tissue pieces were washed with cacodylate buffer (0.1 M) and centrifuged at 10,000 g for 1 minute. The tissue fragments were dehydrated in increasing acetone concentrations (70–95%) after being postfixed in 1% osmium tetraoxide. After being implanted in a solution of embedding medium and propylene oxide, tissue fragments were sectioned using an ultramicrotome. After being stained with uranyl acetate, the sections were examined using a transmission electron microscope (JEM-2100, JEOL).

2.8.4 Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) Assay

The fluorescein-based TUNEL assay method was used for in-situ DNA fragmentation detection. Sequential five m-sized pieces of paraffin-embedded tissue were attached on slides coated with albumin: glycerol (1:1) and left to dry at room temperature. Deparaffinized, rehydrated, and phosphate-buffered saline was used to wash the sections (PBS). Sections were rinsed once more with PBS after being incubated with Proteinase K (20 g/mL in 10 mM Tris-HCl buffer, pH 7.5) for 15 min at room temperature. Sections were then counterstained with DAPI after being treated with a TUNEL reaction solution containing TdT enzyme and Fluorescein-labeled dUTP nucleotides for 60 min at 37 °C. Sections were then viewed under the confocal microscope after being coated with a coverslip (TCS SP5, Leica, Germany).

2.8.5 Protein Expression Analysis in Apoptotic Studies

2.8.5.1 Tissue Homogenization. 10% (w/v) extraction buffer comprising ten mM HEPES (pH 7.4), 0.2 M mannitol, 50 mM sucrose, and one mM EDTA was used to extract the liver and kidney tissues. According to the procedure described by Kaushal *et al.* [27], the tissue homogenate was centrifuged at 13000 g for 15 minutes at 4 °C. The supernatant was immediately removed and kept at –80 °C to investigate the expression of proteins (Caspase 3 and Bcl2).

2.8.5.2 Western Blot Analysis. Western blotting of electrophoresis and transferring was performed as the protocol described. Anti - Caspase 3 IgG (1:1000) antibody, Anti-Bcl-2 IgG (1:500) antibody, or anti-β-actin (1:1000) were considered.

2.9 Statistical Analysis

Using the statistical program "IBM SPSS Statistics 19.0 for Windows", one-way analysis of variance (ANOVA) and Tukey's post hoc test were used to analyze differences between the experimental groups. The data were presented as mean $\pm$ SEM. The statistical magnitude was set at $p \leq 0.05$.

3. Results

3.1 HPTLC Fingerprinting

HPTLC fingerprinting of MFE was done as shown in Fig. 1A, along with various known reference standards like gallic acid, tannic acid, ascorbic acid, naringenin, and quercetin to assess and check their presence in the MFE sample. The plate was developed in the solvent system combination of n-butanol: glacial acetic acid: water in the ratio of (4:1:1 v/v/v). Visualizing of the plate was then followed in absorbance mode λ 254 nm. HPTLC fingerprinting profile of standard was observed with various Rf val-

ues: Naringenin (Rf-0.84), Ascorbic acid (Rf-0.45), Gallic acid (Rf-0.73), Tannic acid (Rf-0.71) and Quercetin (Rf-0.79). Comparison of the MFE fingerprinting Rf value with the standard shows the presence of the standard compound Naringenin (Rf-0.84) and Ascorbic acid (Rf-0.45) in the extract. The Rf value of standard ascorbic acid is 0.45, with a peak area of 2575 in the chromatogram illustrated in Fig. 1B. MFE's Rf value (0.45) coincided with ascorbic acid's peak area of 1538. Standard naringenin has an Rf value of 0.81 and a peak area of 1906. MFE's Rf value (0.82) coincides with the peak area of naringenin, 1455. The presence of both ascorbic acid and naringenin in the MFE sample was confirmed using this analytical approach.

3.2 GLUT4 Protein Expression

The study aimed to analyze how MFE affected the expression of the protein GLUT4 in the skeletal muscle of alloxan-induced diabetic mice. Fig. 2a,b shows that GLUT4 expression was downregulated in diabetic control (0.08 $\pm$ 0.005), with a substantial drop in protein level expression of 0.73 fold compared to standard control (0.33 $\pm$ 0.008). Furthermore, when treated with metformin and MFE, GLUT4 expression was upregulated by 1.8 fold (0.23 $\pm$ 0.01) and 3.25 fold (0.34 $\pm$ 0.005), respectively, compared to the diabetic control.

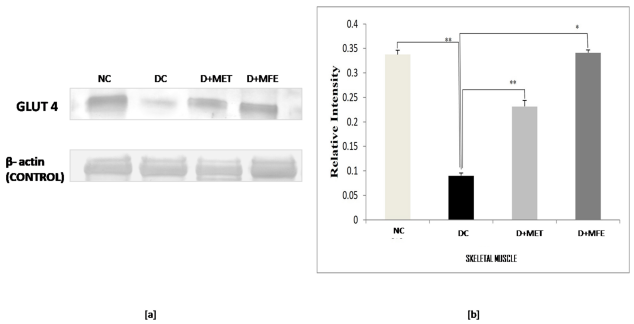

Fig. 2. Comparison of the Glut - 4 protein expression in skeletal muscle between normal control (NC), diabetic control (DC), diabetic mice treated with metformin (D+MET), and diabetic mice treated with MFE (D+MFE). Western blot analysis and corresponding relative intensity after normalization with β-actin (a) and fold differences between experimental groups in skeletal muscle (b). Values are expressed as the mean $\pm$ SEM of triplicate measurements. Significance difference: ** $p < 0.01$ against the normal control and * $p < 0.05$, **$p < 0.01$ against the diabetic control.

3.3 MFE Inhibits Lipid Peroxidation

Lipid peroxidation was assessed by measuring the MDA level in selected tissues of experimental groups. As shown in Fig. 3a, lipid peroxidation was confirmed in diabetic control as a level of MDA increased by 80% (1.30 $\pm$ 0.03) in the liver compared to standard control (0.72 $\pm$ 0.01). Ascorbic acid and MFE-treated groups decreased MDA by 33.3% (0.86 $\pm$ 0.02) and 38.4% (0.80 $\pm$ 0.02), respectively, suggesting reduced lipid peroxidation compared to the diabetic control group. In Fig. 3b, the level of MDA in diabetic control was increased by 66.1% (0.71 $\pm$ 0.008) in the kidney compared to standard control (0.4 $\pm$ 0.008). However, compared to diabetic control, ascorbic acid, and MFE-treated groups exhibited a decrease of MDA by 12.80% (0.61 $\pm$ 0.01) and 28.5% (0.50 $\pm$ 0.003), respectively.

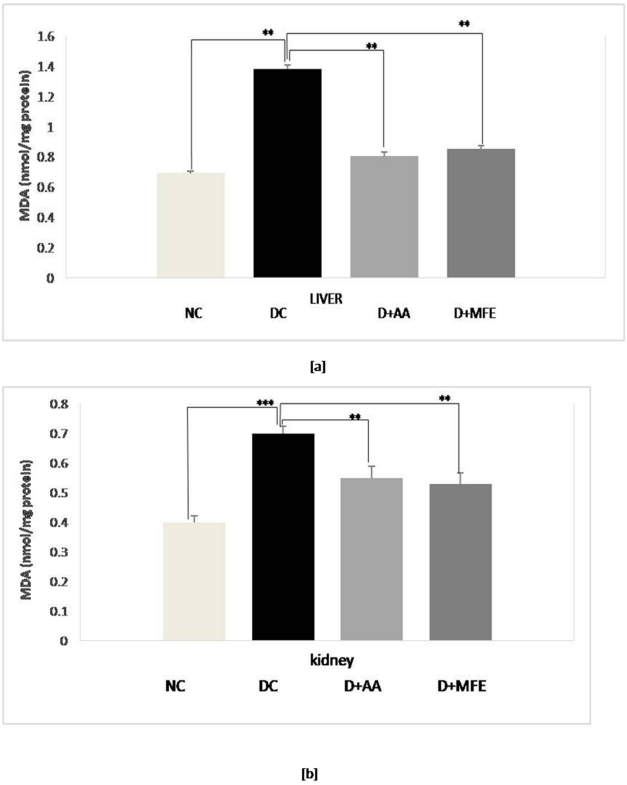

Fig. 3. Mean levels of malondialdehyde (MDA) in the liver tissue (a) and mean levels of MDA in kidney tissue (b) of normal control (NC), diabetic control (DC), diabetic mice treated with ascorbic acid (D+AA), diabetic mice treated with MFE (D+MFE). Values represent the mean $\pm$ SEM. Statistically significant differences: ***$p < 0.001$, **$p < 0.01$ against normal control group and ***$p < 0.001$, and *$p < 0.05$ against diabetic control group.

3.4 MFE Ameliorates Ultrastructural Injuries

The effect of MFE on oxidative stress-mediated cellular changes in different experimental groups' liver and kidney tissues was investigated using ultrastructural techniques. The following are the evaluation's findings.

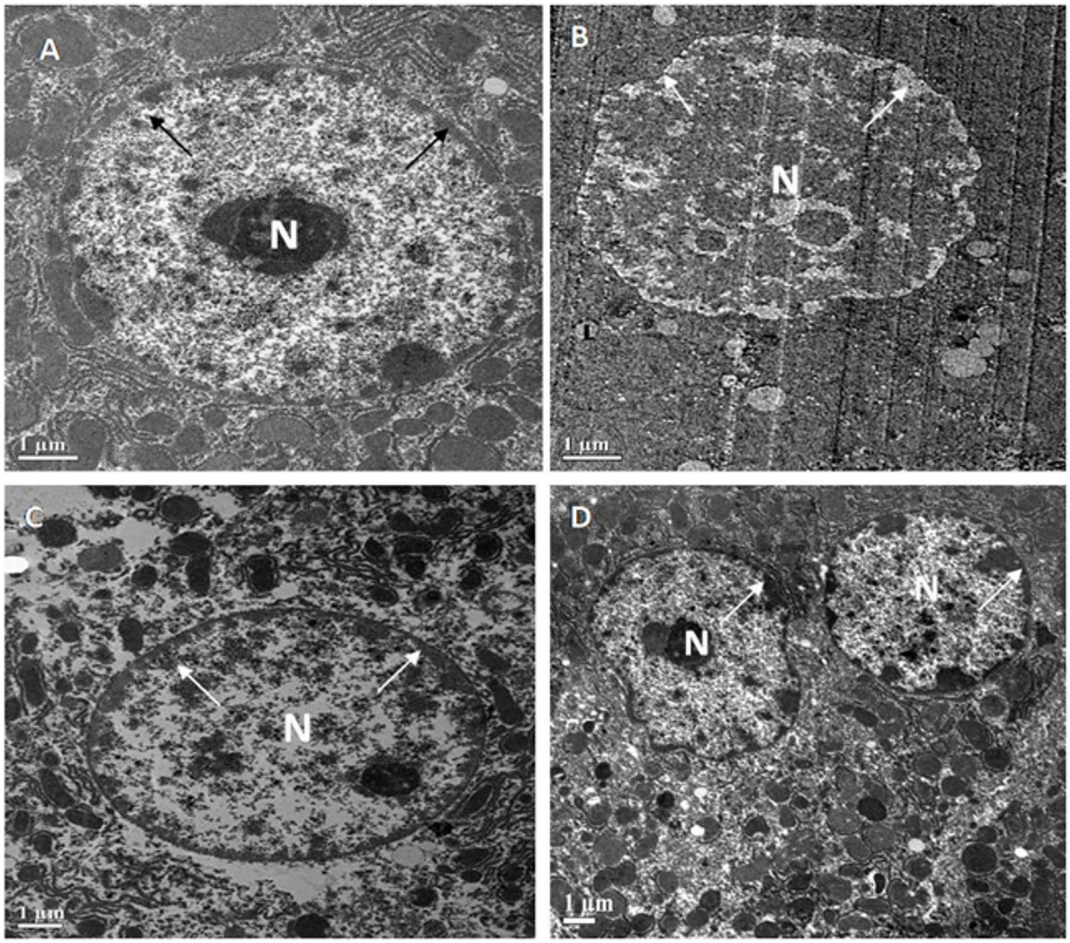

Fig. 4. Representative electron micrographs depict ultrastructural characteristics of hepatocytes in experimental groups containing (A) standard control, (B) diabetic control, (C) diabetic mice treated with ascorbic acid, and (D) diabetic mice treated with the MFE. N, nucleus. Magnification: 2000×.

3.4.1 Liver

The livers of all four groups of Swiss albino mice are depicted in electron micrographs in Fig. 4. A round or oval-shaped nucleus with prominent nucleoli and a symmetrical nuclear membrane was detected in the standard control (A). The diabetic control, on the other hand, had an unevenly shaped nucleus (N) with nearly dissolved nucleoli and an uneven nuclear membrane (B). MFE and ascorbic acid treatment, on the other hand, had an almost identical impact in correcting hepatic abnormalities in both treated groups near normal (oval to round) nucleus (N), with approximately symmetrical nuclear membrane, though some places had uneven nuclear membrane (C,D).

3.4.2 Kidney

Fig. 5 shows an electron micrograph of the kidneys of all four groups of Swiss albino mice. Renal corpuscles with normal extended foot processes (F.P.) lying on the symmetrical glomerular basement membrane (B.M.) were seen in the standard control (A). In diabetic control, however, the ultrastructure of the renal corpuscle was altered, as evidenced by long and aberrant foot processes and abnormal thickening of the glomerular basement membrane (B). Surprisingly, diabetic mice treated with MFE and ascorbic acid had decreased thickening of the glomerular basement membrane (B.M.) and foot processes (F.P.) that were practically normal (C, D).

3.5 MFE Modulates Apoptosis

3.5.1 TUNEL Assay

The TUNEL assay was used to investigate the influence of MFE on apoptosis morphologically using the TUNEL staining method. The percentage of apoptotic cells in the tissues was calculated as follows:

3.5.1.1 Liver. According to the findings in Fig. 6a,b, the diabetic control underwent significant apoptosis, with 37% of cells in the liver tissue being TUNEL-positive, compared to 7.3% in the standard control. Compared to diabetes control, ascorbic acid, and MFE therapy, it has reduced apoptosis by 15% and 17%, respectively, suggesting its usefulness in avoiding *in vivo* oxidative harm.

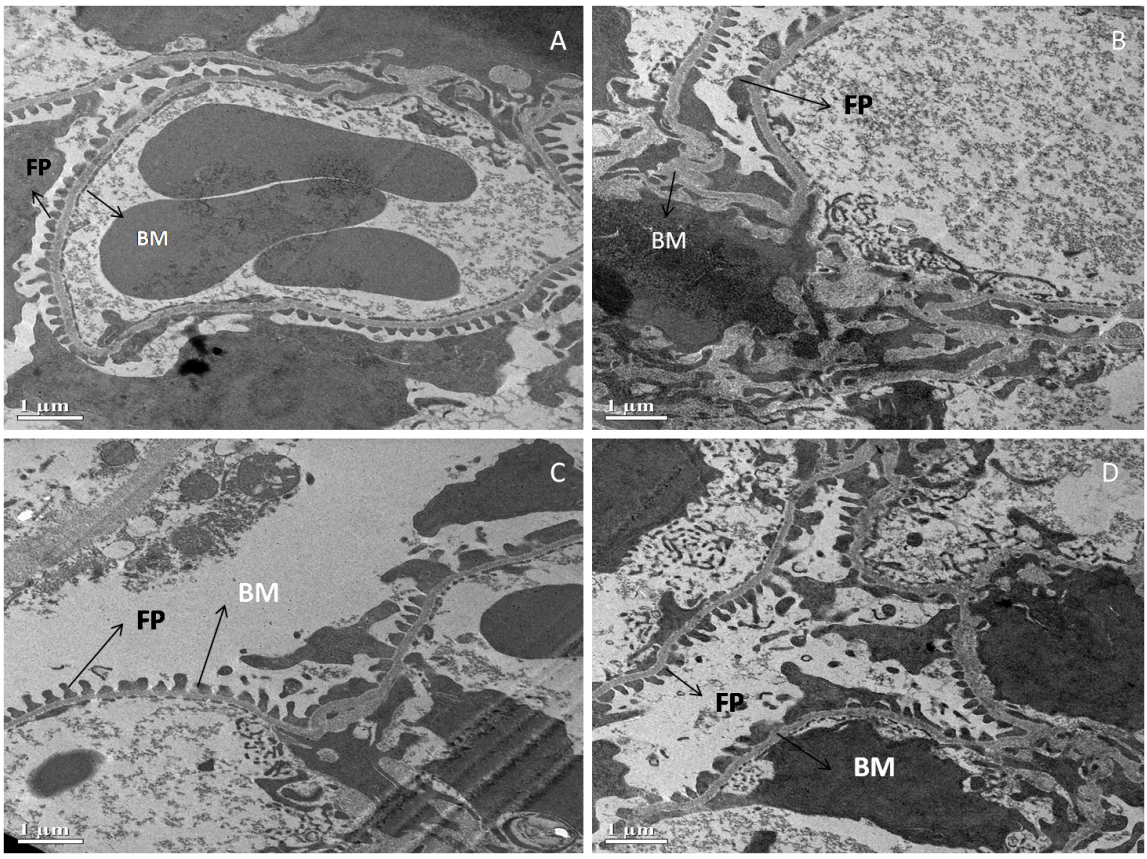

Fig. 5. Representative electron micrographs depict ultrastructural characteristics of renal corpuscle in experimental groups containing (A) standard control, (B) diabetic control, (C) diabetic mice treated with ascorbic acid, and (D) diabetic mice treated with the MFE. B.M., glomerular basement membrane; F.P., foot processes. Magnification: 2000×.

3.5.1.2 Kidney. The percentage of TUNEL-positive cells in diabetic control kidney tissue, as depicted in Fig. 7a,b, was 35%, compared to 6.5% in normal control kidney tissue. When compared to diabetic control in the study, ascorbic acid, and MFE treatment were effective in reducing apoptotic cell death, as TUNEL-positive cells were determined to be 13% and 16%, respectively.

3.5.2 Determination of Bcl-2 and Caspase-3 Protein Expressions

This study aimed to see how MFE affected the expression of Bcl-2 and Caspase 3 proteins in the liver and kidneys of diabetic mice and to compare the results to those of diabetic controls. The following are the outcomes:

3.5.2.1 Liver. **Bcl-2 protein expression*:*** The anti-apoptotic protein Bcl-2 expression was significantly downregulated in the liver tissue of diabetic control, as shown in Fig. 8a,b, with a significant decrease of 0.41-fold (0.18 $\pm$ 0.01) in protein level compared to standard normal control (0.30 $\pm$ 0.01). However, compared to the diabetic control, Bcl2 protein expression was elevated by 0.55-fold (0.28 $\pm$ 0.01) and 0.22-fold (0.22 $\pm$ 0.008) in the ascorbic acid and MFE treatment, respectively.

Caspase 3 protein expression*:* Upregulation of caspase three expressions in liver tissue was seen in diabetic control, as shown in Fig. 8 c,d, with a significant increase of 10.8-fold (0.59 $\pm$ 0.02) in protein level as compared to the standard normal control (0.05 $\pm$ 01). Compared to the diabetic control, ascorbic acid and MFE therapy dramatically reduced caspase three protein expression by 0.74-fold (0.15 $\pm$ 0.005) and 0.67-fold (0.19 $\pm$ 0.003), respectively, when compared to the diabetic control.

3.5.2.2 Kidney. **Bcl-2 protein expression*:*** Downregulation of Bcl2 protein expression in kidney tissue was found in diabetic control, as shown in Fig. 9a,b, with a significant drop of 0.32-fold (0.19 $\pm$ 0.005) in its expression as compared to the standard normal control (0.28 $\pm$ 0.008). Compared to diabetes control, ascorbic acid and MFE therapy enhanced protein expression by 0.47-fold (0.28 $\pm$ 0.01) and 0.21-fold (0.23 $\pm$ 0.005), respectively, compared to the diabetic control.

Caspase 3 protein expression*:* In comparison to the standard normal control (0.11 $\pm$ 0.006), caspase three protein expression in the diabetic control was considerably enhanced up to 2.7 fold (0.41 $\pm$ 0.01) in kidney tissue, as shown in Fig. 9c,d, indicating an increase in protein ex-

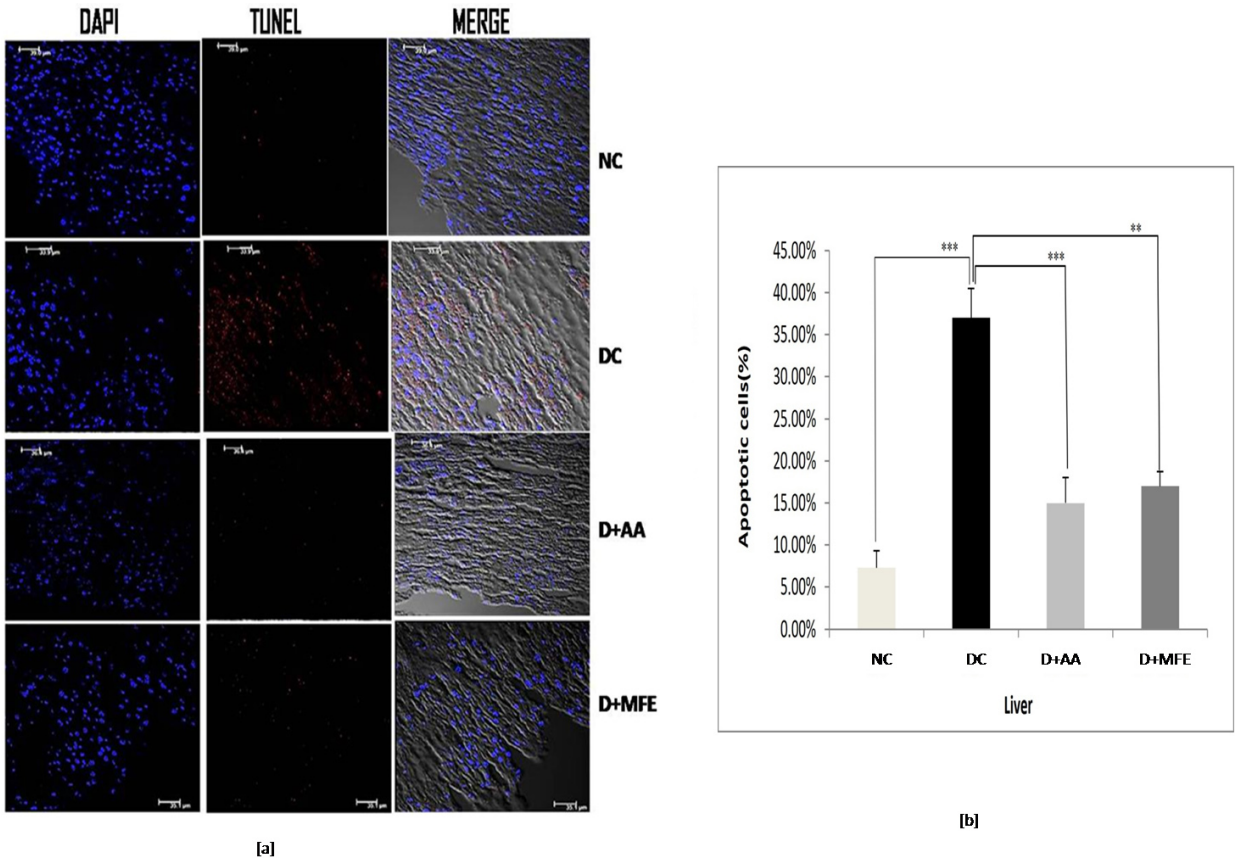

Fig. 6. Confocal microscopic images showing liver tissue sections of normal control (NC), diabetic control (DC), diabetic mice treated with ascorbic acid (D+AA), and diabetic mice treated with MFE (D+MFE). DAPI-stained nuclei are shown in fluorescent blue, whereas TUNEL-positive apoptotic cells are in fluorescent red (a). The corresponding graph shows the percentage of apoptotic cells (b). Values are expressed as the mean ± SEM.Significant differences: ***$p < 0.001$ against normal control and **$p < 0.01$, ***$p < 0.001$ against diabetic control.

pression. However, treatment with ascorbic acid and MFE reduced the expression of caspase-3 protein levels by 0.53-fold (0.19 ± 0.005) and 0.34-fold (0.27 ± 0.008), respectively.

4. Discussion

The pathogenesis of DM and its possible management by existing therapeutic agents having negligible side effects have drawn interest in recent years [33]. Antioxidants generated from plants operate as radical scavengers; therefore, they have gotten much attention. Polyphenols are a class of secondary metabolites that have lately gained popularity due to their potent antioxidant efficacy and ability to protect against chronic illness. Plant phenolics are secondary metabolites generated from phenylalanine and tyrosine extensively dispersed throughout the plant kingdom [34]. HPTLC fingerprinting is an accurate and well-established method for the herbal identification of medicinal plants that may also be used to authenticate and characterize them [35]. HPTLC fingerprinting of MFE revealed the presence of two phytoconstituents in the MFE, ascorbic acid and naringenin, accountable for the pharmaceutical properties of MFE, which implies that ascorbic acid and naringenin perhaps are the active compound present in the extract.

Alloxan (2,4,5,6-tetraoxypyrimidine;2,4,5,6 pyrimidinetetrone) is an oxygenated pyrimidine derivative found as alloxan-hydrate in aqueous solution with diabetes-inducing effects when administered intravenously, intraperitoneally, or subcutaneously in rodents [36]. Alloxan induces insulin-dependent diabetes, which culminates in the selective necrosis of beta cells by selectively blocking the production of glucose-induced insulin through specific inhibition of the enzyme glucokinase and by its capacity to generate ROS generation [37].

Under diabetes mellitus conditions, the glucose transporter protein GLUT4 in the cell membrane is reduced due to the lack of insulin sensitivity. This leads to decreased cell glucose uptake or hyperglycemia [38]. Insulin regulates the presence or absence of GLUT4 on the plasma membrane's cell surface. The effect of MFE on the expression of GLUT4 protein in the skeletal muscles of diabetic mice was studied. The study found that GLUT4 expression was downregulated in diabetic mice, indicating

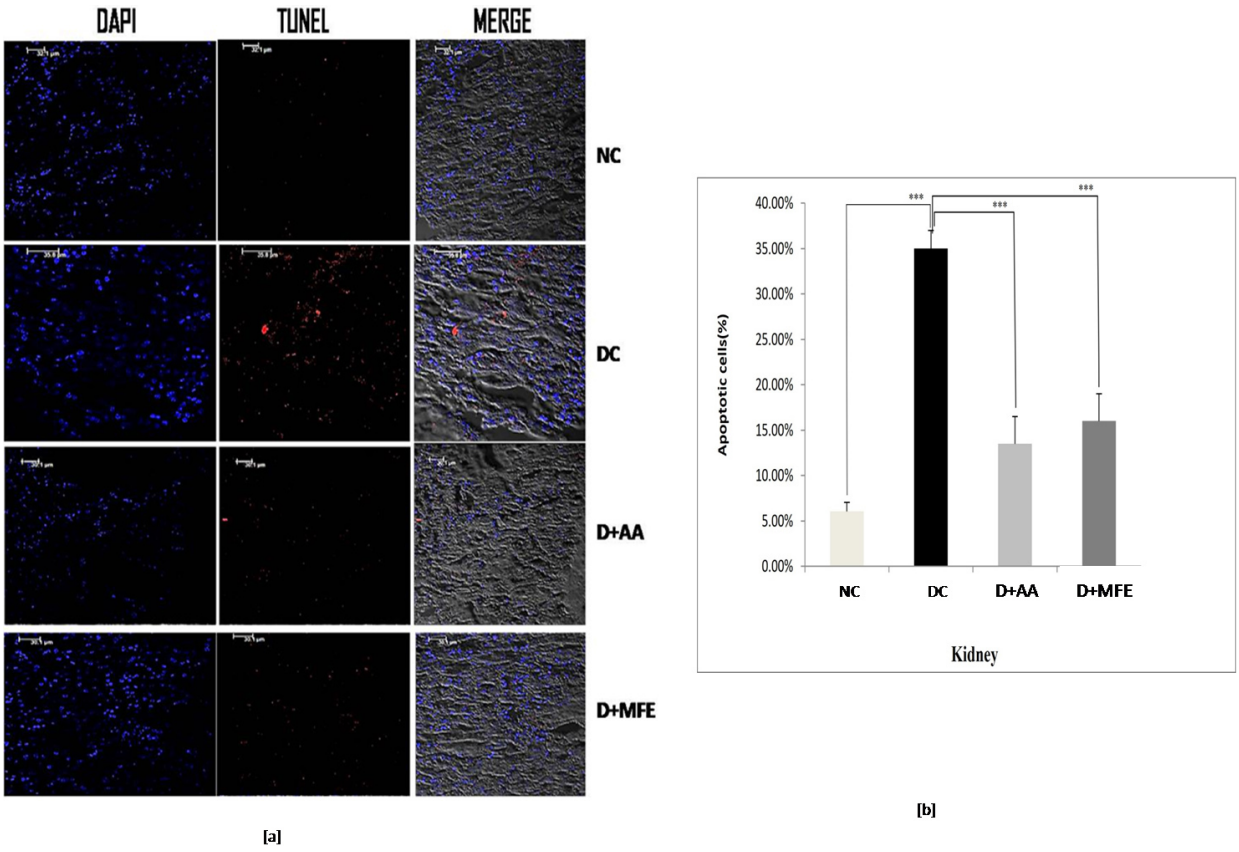

Fig. 7. Confocal microscopic images showing kidney tissue sections of normal control (NC), diabetic control (DC), diabetic mice treated with ascorbic acid (D+AA), and diabetic mice treated with MFE (D+MFE). DAPI-stained nuclei are shown in fluorescent blue, whereas TUNEL-positive apoptotic cells are in fluorescent red (a). The corresponding graph shows the percentage of apoptotic cells (b). Values are expressed as the mean ± SEM. Significant differences:*** $p < 0.001$ against normal control and ***$p < 0.001$ against diabetic control.

a relative insulin deficit. In contrast, after MFE therapy, GLUT4 expression was shown to be increased and equivalent to GLUT4 expression in metformin-treated diabetic mice, indicating an increase in insulin availability in the treated groups. Increased GLUT4 protein levels would increase glucose absorption in skeletal muscle, assisting in treating hyperglycemic conditions. MFE was discovered to have an anti-hyperglycemic effect against alloxan-induced hyperglycemia, according to the study's findings.

High glucose concentrations cause the tricarboxylic acid cycle (TCA cycle) to overproduce electron donors, increasing the mitochondrial proton gradient and the production of mitochondrial superoxide [39,40]. An increase in free radicals then causes oxidative stress-induced lipid peroxidation. This process causes a decrease in cell membrane fluidity and changes the activity of membrane-bound enzymes and receptors, resulting in membrane malfunction [41]. Lipid peroxidation attacks the lipids containing carbon-carbon double bond(s), such as polyunsaturated fatty acids (PUFAs). The present study also showed a significant elevation of MDA levels in all three liver and kidney organs of alloxan-induced diabetic mice, notably due to the formation of increased ROS in the tissues. Intraperitoneal administration of MFE to diabetic mice at a dose of 350 mg/kg b.w showed a significant reduction in MDA level. It implies the antioxidative nature of MFE to stop free radical chain reaction, parallel to the activity exhibited by intraperitoneal administration of ascorbic acid at a dose of 50 mg/kgb.w.

Ultrastructural examination of diabetic liver and kidney tissues revealed unhealthy tissues. MFE administration produced positive results in restoring hepatocyte and kidney tissues, with effects comparable to ascorbic acid. As a result of the antioxidant activity exerted by the MFE, it was deduced from the ultrastructural experiments that it demonstrated a good response in repairing tissue architecture.

It has been established that under hyperglycemia-induced oxidative stress, various cell types undergo a ubiquitous phenomenon known as apoptosis (cell death) [42]. A characteristic feature of apoptotic cell death is DNA strand breakage. This study used the TUNEL assay to detect apoptotic cells because the TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. The study found a substantial number of TUNEL-positive cells and

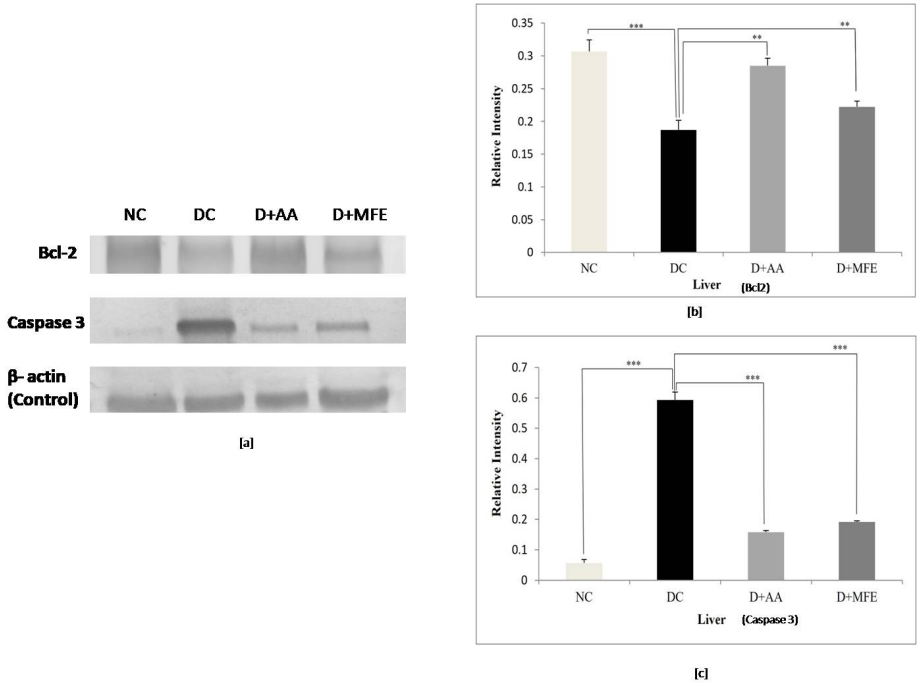

Fig. 8. Comparison of Bcl-2 and Caspase 3 protein expressions in the liver between normal control (NC), diabetic control (DC), diabetic mice treated with ascorbic acid (D+AA), and diabetic mice treated with MFE (D+MFE). Western blot analysis of Bcl-2 and Caspase 3 protein expression, corresponding relative intensity after normalization with β-actin (a), and fold differences between experimental groups in the liver (b,c). Values are expressed as the mean $\pm$ SEM of triplicate measurements; Significance difference: *** $p < 0.001$ against the standard control and ** $p < 0.01$ against the diabetic control.

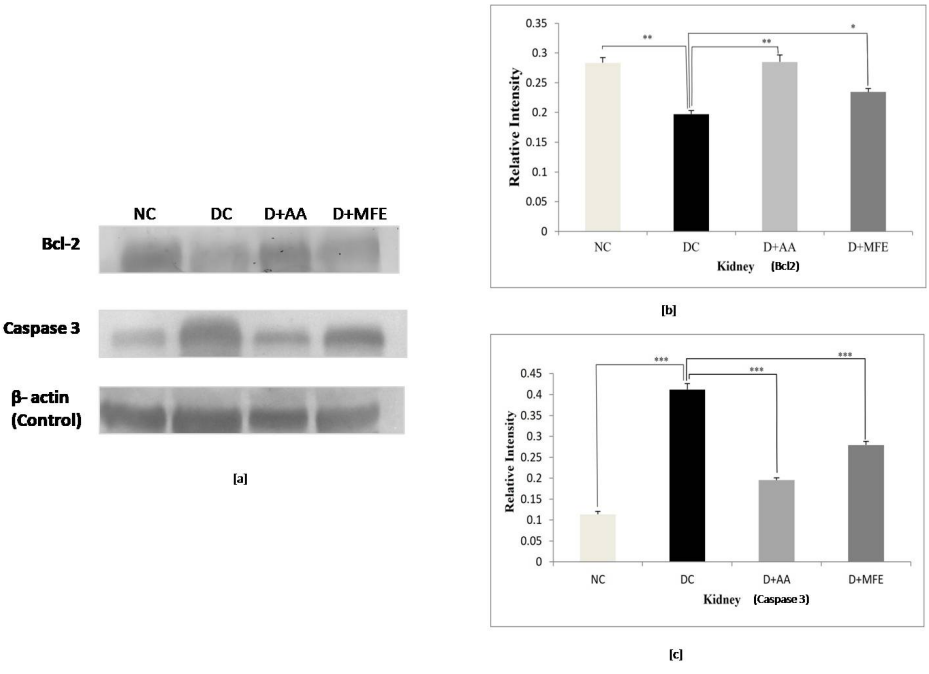

Fig. 9. Comparison of Bcl-2 and Caspase 3 protein expressions in kidneys between standard control (NC), diabetic control (DC), diabetic mice treated with ascorbic acid (D+AA), and diabetic mice treated with MFE (D+MFE). Western blot analysis of Bcl-2 and Caspase 3 protein expression, corresponding relative intensity after normalization with β-actin (a), and fold differences between experimental groups in the kidney (b,c). Values are expressed as the mean $\pm$ SEM of triplicate measurements; Significance difference:*** $p < 0.001$ against the standard control and ** $p < 0.01$ against the diabetic control.

a 37% and 35% increase in apoptotic cells in the liver and kidney, respectively, in the diabetic state caused by alloxan. However, administration of ascorbic acid and MFE lowered the number of TUNEL-positive cells in diabetic mice's liver and kidney tissues, indicating reduced apoptosis. As a result, the findings of the TUNEL assay may be corroborated by the study's western blot analysis of apoptosis-associated proteins.

To observe the effect of MFE apoptotic cell death, caspase three and Bcl-2 protein expression in mice's hepatocytes and renal tissues were studied. In the current study, diabetic mice had lower Bcl-2 expression and higher caspasethree3 expressions. Diabetic mice treated with ascorbic acid and MFE showed reduced caspase three expressions and increased Bcl-2 expression. Thus, the study's TUNEL assay and western blot analysis results indicate the anti-apoptotic nature of MFE against hyperglycemia-induced oxidative stress apoptosis. However, ascorbic acid was taken as a positive control for conducting all the experiments. A similar outcome was observed, implying that ascorbic acid could be the active compound, as its presence was confirmed in the HPTLC method.

Phytochemicals are essential components, specifically for their role as antioxidants [43]. ROS generated during hyperglycemic conditions are molecules that are unstable in nature and thus create oxidative stress. Antioxidants neutralize these unstable molecules by losing some of their own electrons. HPTLC of MFE revealed the presence of two phytochemicals, ascorbic acid, and Naringenin. Ascorbic acid, an antioxidant, works by donating a single reducing equivalent, forming monodehydroascorbate, which is radical in nature, and monodehydroascorbate reacts preferentially with radicals instead of non-radical compounds [44]. Ascorbic acid has also been proven to regenerate the antioxidant form of vitamin E to protect the membrane and hydrophobic compartments from damage. Further, a resident of hydrophobic compartments, reduced coenzyme Q, interacts with vitamin E to regenerate its antioxidant form [45]. Naringenin is a flavonoid that prevents injury caused by free radicals, where radicals oxidize flavonoids. In the process, the radical becomes a more stable and less-reactive form. The high reactivity of the hydroxyl group of flavonoids makes radicals inactive. This potential mechanism of the phytochemicals in MFE could be the combined effect in its therapeutic diabetes treatment. The confirmed mechanism needs to be studied in the future [46].

5. Conclusions

In conclusion, it can be inferred from the study that DI extract possesses free radical scavenging properties giving insights regarding its antioxidative and anti-apoptotic effects in alloxan-induced diabetic mice. The bioactivity of DI in the study presents opportunities as a potential source of therapeutic agents for treating diabetes and its associated complications.

Abbreviations

DM, Diabetes mellitus; ROS, Reactive oxygen species; Apaf-1, Apoptosis protease activating factor-1; MFE, Methanolic fruit extract of *Dillenia indica*; FBGL, Fasting blood glucose levels; HPTLC, High-Performance Table Liquid Chromatography; GLUT 4, Glucose Transporter 4; IEC, Institutional Ethics Committee; SDS, Sodium dodecyl sulfate; TBS, Tris-buffered saline; TTBS, Tween Tris-buffered saline; HRP, hydrogen peroxidase; TEM, Transmission Electron Microscope; TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

Availability of Data and Materials

All applicable data and materials are included in the manuscript.

Author Contributions

PS and JB designed the study, made the first draft, and updated the manuscript, data curation, and experiment. SM designed the study, made the first draft, updated the manuscript, a reviewed the final draft. DS designed the study and updated the manuscript. SB designed the study, made the first draft, data curation, and reviewed the final draft, critically reviewed and approved the final draft, Funding acquisition. AH updated the manuscript and data curation. SR made the first draft and critically reviewed the final draft. NSS updated the manuscript and data curation. SH updated the manuscript, critically reviewed it, and approved the final draft. NS updated the manuscript and data curation. AGA reviewed and made the first draft, updated the manuscript, reviewed the final draft, data curation, reviewed the final draft funding acquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study protocol was approved by the Institutional Ethics Committee (Animal model), North Eastern Hill University, Shillong, Meghalaya, India.

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Conflict of Interest

The authors declare no conflict of interest.

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