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ORIGINAL ARTICLE

Therapeutic Potential of *Boerhavia diffusa* L. against Cyclosporine A-Induced Mitochondrial Dysfunction and Apoptosis in Madin–Darby Canine Kidney Cells

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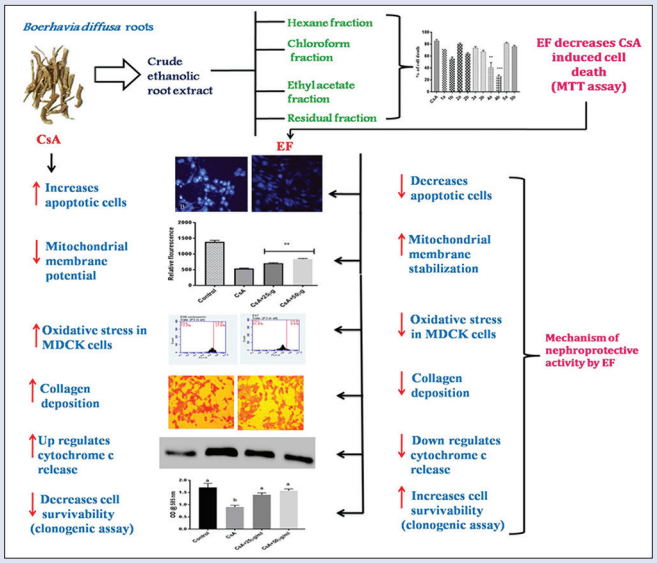
ABSTRACT

Introduction: Cyclosporine A (CsA), a calcineurin inhibitor, causes chronic nephrotoxicity during organ transplantation. **Objective:** The aim is to investigate the effect of ethyl acetate fraction (EF) of *Boerhavia diffusa* on CsA-induced cell damage due to apoptosis and reactive oxygen species (ROS). **Materials and Methods:** Madin–Darby Canine Kidney (MDCK) cells were treated with CsA alone and CsA plus EF (25 and 50 µg/ml). The mechanism of attenuation of apoptosis and ROS by EF was studied using various experiments. **Results**: EF protected the MDCK cells from CsA-induced apoptosis and cell cycle arrest at G_0/G_1 and sub G_0/G_1. The protective effect was further confirmed by cytochrome c translocation into cytoplasm and caspase 3 release. In addition, CsA-induced ROS production was abolished by the antioxidant potential of EF, which could be attributed to the polyphenol content. The decrease in ROS generation was confirmed by DCF-DA staining, LPO, and nitric oxide release in the MDCK cells. Moreover, the accumulation of collagen was decreased in EF-treated groups and increased the survivability of cells. The gas chromatography–mass spectrometry analysis revealed the presence of alkaloid and phenolic compounds. To the best of our knowledge, this is the first report showing the presence of alkaloid Carnegine and N-Benzyl-2-phenethylamine in this plant. **Conclusion:** Hence, it could be justified that EF-protected MDCK cells against CsA stimulated renal cell damage by attenuating apoptosis and ROS generation. Further isolation of compound has to be carried out to explore the effectiveness of EF as nephroprotective drug.

Key words: Antioxidant activity, collagen 1, cyclosporine A, cytochrome c, gas chromatography–mass spectrometry analysis, intrinsic apoptotic pathway

SUMMARY

- Cyclosporine A (CsA) induced nephrotoxicity is the common side effect that occurs during solid organ transplantation.
- In general, ROS induced toxicity has been significantly reduced by plant based drugs.
- *Boerhavia diffusa* is a potent diuretic agent used in Ayurvedic treatment since ancient times.
- Therefore, the current study evaluated the protective role of *B. diffusa* against CNI-induced nephrotoxicity by attenuating apoptosis and ROS production.



Abbreviations used: CsA: Cyclosporine A; EF: Ethyl acetate fraction; CE: Crude extract; HF: Hexane fraction; CF: Chloroform fraction; RF: Residual fraction.

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INTRODUCTION

Cyclosporine A (CsA), a calcineurin inhibitor, improves the success rate in solid organ transplantation because of its immunosuppressive properties. The long-term usage of CsA causes chronic nephrotoxicity, which leads to irreversible lesions, namely, striped interstitial fibrosis, tubular atrophy, and hyalinosis of afferent arterioles.[1] Despite many mechanisms involved in CsA-induced nephrotoxicity, a significant role is contributed by reactive oxygen species (ROS).[2] CsA-induced mitochondrial ROS could affect cellular functions such as intracellular calcium control, gluconeogenesis, and fatty acid synthesis.[3] In addition, CsA stimulates apoptosis by both caspase-dependent loss of mitochondrial membrane potential and caspase-independent release of cytochrome c.[4]

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Boerhavia diffusa (Nyctaginaceae family) is a popular herb used in Asian medicine. The roots have diuretic, laxative, and expectorant properties. It has been used for the treatment of dyspepsia, jaundice, spleen enlargement, abdominal pain, and as an anti-stress agent.[5,6] Pharmacological studies have revealed the presence of numerous phytoconstituents with therapeutic attributes such as antifibrinolytic, anticonvulsant, antibacterial, analgesic and anti-inflammatory property, hepatoprotective activity, immunomodulatory activity, and anti-proliferative properties.[7] Thus, the current study explored the beneficial role of ethyl acetate fraction (EF) of *B. diffusa* against CsA-induced oxidative injury using Madin–Darby Canine Kidney (MDCK) cells as model system to neutralize CsA-induced nephrotoxicity.

MATERIALS AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), propidium iodide (PI), bisBenzimide (Hoechst staining), and rhodamine 123 were purchased from Sigma Aldrich, India. Cytochrome c was purchased from Santa Cruz Biotech. CsA was purchased from Selleck chemicals.

Cell culture

MDCK epithelial cells cell line was procured from the National Centre for Cell Sciences and maintained in DMEM supplemented with 10% FBS in a humidified 5% CO_2 incubator at 37°C.

Plant material

B. diffusa roots were collected near Chennai, Tamil Nadu, India. Prof. P. Jayaraman, Ph. D, Director of National Institute of Herbal Science, Chennai, Tamil Nadu, India, authenticated the plant and a voucher specimen (PARC/2011/582) is maintained for further reference in Department of Biomedical Sciences, Sri Ramachandra University. The dried roots were coarsely ground and stored at room temperature for further use.

Extraction and fractionation

The coarsely powdered root material was extracted using 70% ethanol for 72 h by cold percolation method. Following the extraction, it was filtered and concentrated in a rotary evaporator. The dried crude extract (CE) was dissolved in water and fractionated successively using different solvents based on increasing polarity such as hexane fraction, chloroform fraction, and EF. The remaining water fraction was considered as rest fraction.[8]

Nephroprotective activity of various fractions

Cell viability assay

Cell viability was performed using MTT assay. Briefly, 5×10^3 cells were seeded in 96 well plate and incubated in 5% CO_2 incubator. After confluency, the cells were treated with 10 µM of CsA alone and along with CE and the various fractions (10, 25, 50, 75, and 100 µg/ml). Following 24 h of incubation period, 20 µl of MTT (5 mg/ml) was added and incubated for 2 h. The formazan crystal formed was dissolved by DMSO and the absorbance was measured at 570 nm.[9]

Lactate dehydrogenase assay

Cells were treated with CsA (10 µM) alone or in combination with 25 and 50 µg/ml of EF and incubated for 24 h. After incubation, LDH released in the medium was measured at the absorbance of 440 nm. To 1 ml of the buffered substrate (lithium lactate dissolved in glycine buffer), 0.1 ml of conditioned media was added and kept in incubator at 37°C for 5 min. Then, 0.2 ml of NAD + solution was added, mixed gently, and incubated at 37°C for 15 min. To this, 1 ml of DNPH reagent was added and further incubated for 15 min.[10] Finally, 10 ml of sodium hydroxide (0.4 N) was added and absorbance was read at 440 nm. Standard graph was prepared using sodium pyruvate. The amount of LDH released in the medium was calculated using the formula

LDH activity = OD of unknown/OD of known × standard concentration = µg of Lactate liberated/ml of conditioned media.

Morphological staining for apoptosis

After treatment, the cells were fixed with 4% formaldehyde, stained with 10 µg/ml of Hoechst 33342, and incubated for 10 min to stain the nuclei. Cells were observed under fluorescence microscopy and the number of apoptotic cells were calculated.[11]

Cell cycle analysis by fluorescence-activated cell sorting (FACS)

For FACS analysis, control and treated cells were washed with ice-cold phosphate-buffered saline (PBS). The cells were scraped using rubber policeman and centrifuged at 2000 rpm for 10 min. Then, cells were mixed with 500 µl of hypotonic solution (1% sodium citrate, 0.04% RNAse, 0.05% PI, and 3%Triton X-100) and the distribution of cells in different phase was determined by C6 Accuri (BD) flow cytometer.[12]

DNA fragmentation

After 24 h incubation, the cells were trypsinized and centrifuged at 2500 rpm for 15 min. To the pellet, SE buffer (25 mM Na_2EDTA, 200 mMNaCl, pH 8), 5 µl proteinase K (50 µg/ml), and 25 µl of 10% SDS were added and incubated in water bath at 37°C for overnight. After incubation, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well, and spun at 10,000 rpm for 15 min at room temperature. The upper aqueous layer was collected separately, and 2.5 volume of chilled ethanol was added and the tube was inverted gently for several hours. The DNA was transferred into a tube containing 1 ml of 70% ethanol and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was discarded and the pellet was air-dried for 3 h. 1X TE buffer was added to dissolve the pellet and stored at 4°C. DNA samples were electrophoresed on a 1.5% agarose gel for 1 h and 30 min at 70 V. Finally, the gel was examined under UV light to determine apoptotic DNA fragmentation.[13]

Annexin V stain and propidium iodide uptake

Annexin V and PI uptake staining were determined using commercial apoptosis assay kit. Briefly, following treatment, the floating and scraped cells were pooled by centrifuging at ×1000 g for 5 min. The cells were washed twice with PBS, and resuspended in binding buffer (100 µl). The single cell suspension was incubated with 5 µl of fluorescein isothiocyanate (FITC)-labeled Annexin V and 5 µl of PI for 30 min in the dark. Subsequently, the cells were washed twice with binding buffer and the samples were analyzed using flow cytometry (FACScan, BD Biosciences). A total of 10,000 events were calculated for each sample.[14]

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was assessed using the fluorescent dye Rhodamine B. Following treatment, Rhodamine dye was added in each well and incubated for 30 min. After incubation, the cells were washed with ice-cold PBS and analyzed by flow cytometry.[15]

Cytochrome c release

The drug-treated cells were lysed in RIPA buffer containing 1X protease inhibitor cocktail, and protein concentrations were estimated by Lowry's method.[16] Cell lysate (50 µg) were electrophoresed in 12% SDS

polyacrylamide gel and then transferred onto PVDF membranes. The membranes were incubated with primary antibodies against cytochrome c (1:2000) in Tris-buffered saline. The washed PVDF membranes were incubated with HRP conjugated antimouse IgG (1:5000) and Goat anti-rabbit IgG (1:5000). Protein bands were detected using chemiluminescence system (ECL kit) and quantified in ChemiDoc XRS Imaging System, Bio-Rad (USA).

Measurement of caspase 3 activity

Caspase 3 activity was measured using EnzChek Caspase Assay Kit according to the manufacturer's instruction. Briefly, the treated cells were lysed using lysis buffer and incubated for 30 min. After incubation, 50 µl of 1x lysis buffer, 1 µL of the 1 mMAc-DEVD-CHO inhibitor stock solution was added and incubated for 10 min. About 10 µL of the 5 mM Z-DEVD–R110 substrate was added and incubated again for 30 min. The fluorescence was measured at 496/520 nm using excitation and emission filters.

Measurement of reactive oxygen species

To measure the level of ROS, the drug-treated cells were incubated with 20 µM DCFH-DA for 30 min. Following which the floating and adherent cells were centrifuged at 1000 rpm for 10 min and a minimum of 10,000 cells were subjected to flow cytometry using a FACSCalibur.[15]

Measurement of lipid peroxidation

After drug treatment, the cells were scraped and the cells were washed twice with the PBS containing calcium and magnesium. The cells were lysed in 520 µl of 1.15% of potassium chloride for 30 min and centrifuged at 5000 rpm. About 500 µl of cell supernatant was incubated with 2 ml of TCA (10%) at 100°C. The samples were cooled and centrifuged at 1000 rpm for 10 min and 2.5 ml of the supernatant was mixed with 1 ml of 0.8% TBA and incubated for 15 min at 100°C. The absorbance was measured at 532 nm and at 600 nm.[17]

Nitric oxide release

Nitrate was reduced to nitrite and determined with Griess reagent by adding sulfanilic acid and naphthylenediamine (1 mM in the assay). The absorbance at 548 nm was compared with a standard of $NaNO_2$.[18]

Collagen staining using direct red 80

Collagen was measured using direct red 80. Direct red dye (0.1%) was prepared in saturated picric acid. The cells were washed with PBS and fixed with Bouin's fluid (15 ml saturated aqueous picric acid with 5 ml of 35% formaldehyde and 1 ml of glacial acetic acid) for 1 h. After incubation, the cells were washed with PBS, air-dried, and then 1 ml of direct red dye reagent was added kept for 1 h under mild shaking on a microplate shaker. Excess dye from the cells was washed with 0.01 N HCL. The stained cells were photodocumented and the dye was dissolved using 1 ml of 0.1 N NaOH and incubated for 30 min. OD was measured at 550 nm against 0.1NNaOH as a blank and OD/10^5 cells were plotted.[19]

Clonogenic assay

Clonogenic assay is an *in vitro* cell survival assay to determine cellular reproductive capability consequent to treating with ionizing radiation. After drug treatment, cells were washed with PBS and trypsinized to get a single cell suspension. The number of cells was counted using trypan blue staining and hundred cells were cultured in a drug-free complete medium. After 2 weeks, the medium was removed and washed the cells with PBS. The colonies were fixed with 10% neutral buffered formalin solution for 15–30 min. Then, the cells were stained with crystal violet (0.01%) and kept for 30 min. The crystal violet solution was removed and washed with distilled water to remove the excess dye. Finally, the intracellular dye was eluted with 50% sodium citrate 0.1 M (pH 4.2) and the OD was measured at 595 nm.[20]

Gas chromatography–mass spectrometry analysis

Gas chromatography–mass spectrometry (GC-MS) analysis was carried out on GC-MS-5975C (AGILENT) using the following conditions. The carrier gas was helium and 2 µL of the sample was injected at a constant flow rate 1.51 ml/min. The column temperature was programmed to 70°C with an increase of 10°C/min reaching a maximum 300°C. The mass spectra were obtained through ionization energy of 70 eV in the EI mode. Total GC-MS running time was 30 min. The organic compounds were identified by comparison of mass spectra with the inbuilt libraries the National Institute of Standard and Technology (NIST-11).

Statistical analysis

Results were expressed as mean ± standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's *post hoc* multiple comparison tests using the GraphPad Prism 7 program (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Ethylacetate fraction increases the cell viability

To study the nephroprotective role of *B. diffusa*, MDCK cell line (distal tubular epithelial cells of canine origin) was used. Initially, CE and various fractions were screened for nephroprotective activity against CsA. The toxicity induced by CsA (10 µM) in MDCK cells was reduced by EF effectively when compared to CE and other fractions [Figure 1a]. Cells exposed to CsA showed increased cell death after 24 h. The cell death was markedly reduced with the simultaneous treatment with EF at 50 µg/ml. Phase-contrast photomicrographs after 24 h showed that cell density was decreased in CsA-treated groups when compared to control. Cells exposed to CsA and EF showed good cell morphology with increased population of live cells [Figure 1b].

CsA also damages the integrity of outer membrane as evidenced by the increase of LDH release in the medium but EF decreased the LDH release into the medium dose dependently ($P < 0.001$). This result showed that EF improves the cell viability by retaining the plasma membrane integrity [Figure 2a]. To study the role of CsA-inducing apoptosis, the cells were stained with Hoechst 33342 dye [Figure 2b]. Control cells appeared to be intact, and the nuclei exhibited reduced in bright blue fluorescence, but CsA-treated cells exhibited the apoptotic morphology such as cell shrinkage, fragmented segregated bodies. Further EF-treated cells showed improved integrity of cells, thereby reduced apoptotic morphology.

Ethylacetate fraction inhibits cyclosporine A-induced apoptosis and necrosis

Cell distribution in various phases of cell cycle was explored using flow cytometry. The results revealed that CsA increases the number of cells in sub G_0/G_1 and G_0/G_1 phase and concomitant cell depletion was observed in S phase. This showed that CsA induces both cell cycle arrest and hence cell death in MDCK cells. However EF-treated group showed gradual decrease in the number of cells in sub G_0/G_1 and G_0/G_1 phase confirming that EF could rescue the cells from cell cycle arrest and cell death [Table 1].

Number of cells in sub G_0/G_1 phase was higher in CsA-treated groups which confirms that cell death was induced by CsA. To study whether the

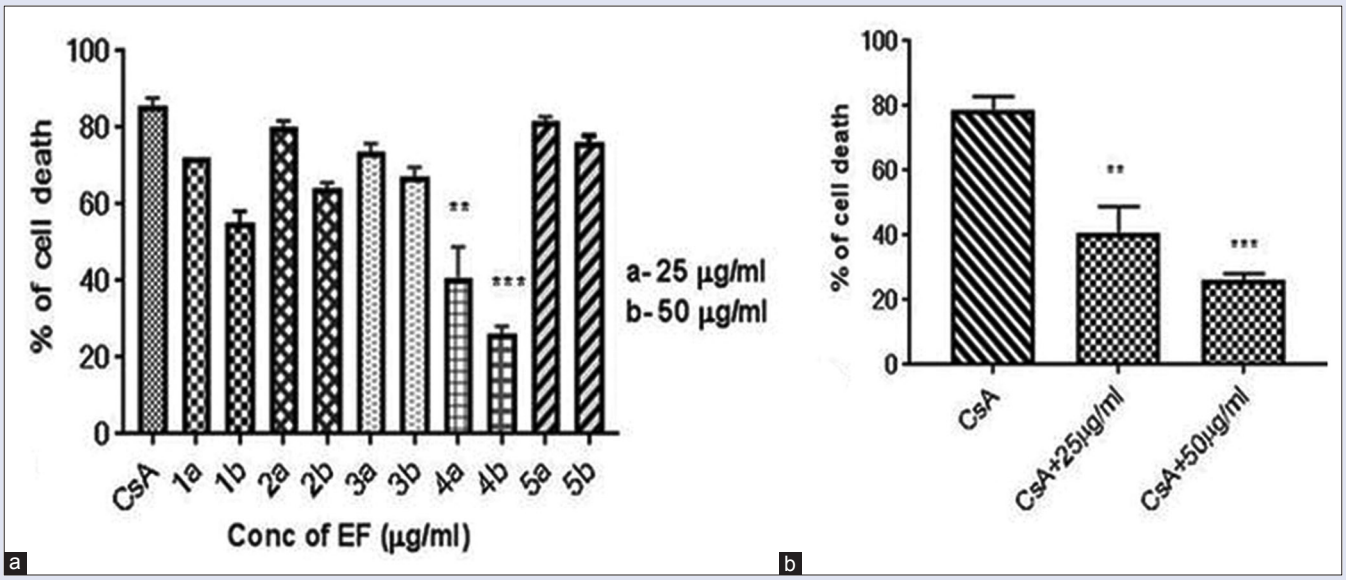

Figure 1: The viability of Madin–Darby canine kidney cells treated with *Boerhavia diffusa* against cyclosporine A-induced nephrotoxicity. (a) Effects of crude extract and different fractions. (b) The effect of ethyl acetate fraction on cyclosporine A-induced cell death (at full page width)

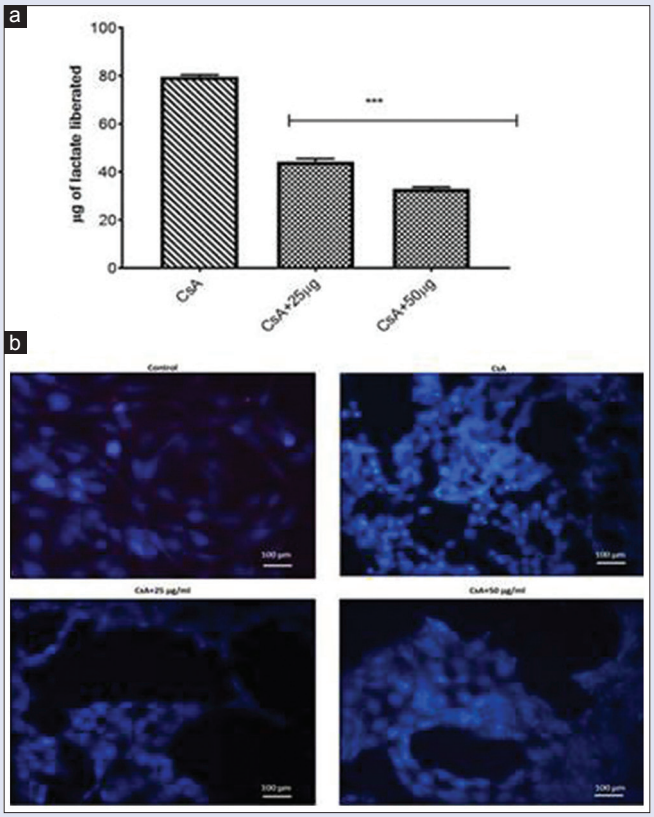

Figure 2: (a) Effect of ethyl acetate fraction on cell membrane integrity. (b) Treated cells stained with Hoechst observed under fluorescent microscope (×200). Cyclosporine A-treated cells showed apoptotic morphology, but concurrent treatment with ethyl acetate fraction showed less apoptotic morphology (at column width)

CsA-induced cell death was due to apoptosis or necrosis and to examine the protective effect of EF the cells were subjected to Annexin-V-FITC and PI staining. CsA at the concentration of 10 µM showed increased number of late apoptotic cells (annexin and PI positive cells) and necrotic cells (PI-positive cells). However, significant decrease in the PI-positive cells ($P < 0.0001$) could be observed with increasing concentration of EF [Figure 3a and b]. Furthermore, laddering of DNA was substantially less in EF-treated group compared to CsA group [Figure 3c].

Table 1: The effect of ethyl acetate fraction on cyclosporine caused cell cycle arrest and cell death. Data represented as mean±standard deviation (*n*=3)

Treatment	% of cells in each phase in the cell cycle and cell death in sub G_0/G_1 phase			
	Sub G_0/G_1	G_0/G_1	S	G_2/M
Control	0.00±0.08	44.48±0.12	17.56±0.12	25.56±0.08
EF 25 µg/ml + CsA	10.81±0.03	47.76±0.05	12.36±0.37	23.81±0.32
EF 50 µg/ml + CsA	8.8±0.07	45.01±0.2	15.76±0.46	25.56±0.17
CsA	23±0.1	58.09±0.31	9.56±0.21	15.61±0.54

Ethylacetate fraction reduces the mitochondrial membrane potential and inhibits apoptosis

CsA decreased the incorporation of rhodamine in the mitochondria, which indicates the disruption in mitochondrial membrane potential [Figure 4a]. However, EF significantly increased the rhodamine incorporation, thus preventing the disruption of mitochondrial membrane ($P < 0.05$). As a result of mitochondrial damage, release of cytochrome c in the cytosol was observed but EF attenuated the expression of cytochrome c [Figure 4c and d]. Caspase 3 released as a continuum of cytochrome c translocation which is an executioner step of apoptosis was decreased by EF ($P < 0.001$) in the treatment group [Figure 4b].

Ethylacetate fraction reduces the oxidative stress in cyclosporine A-treated cells

Intracellular ROS level was calculated using flow cytometry after staining the cells with the fluorescent dye DCFH-DA. When compared with control, the percentage of fluorescent cells was

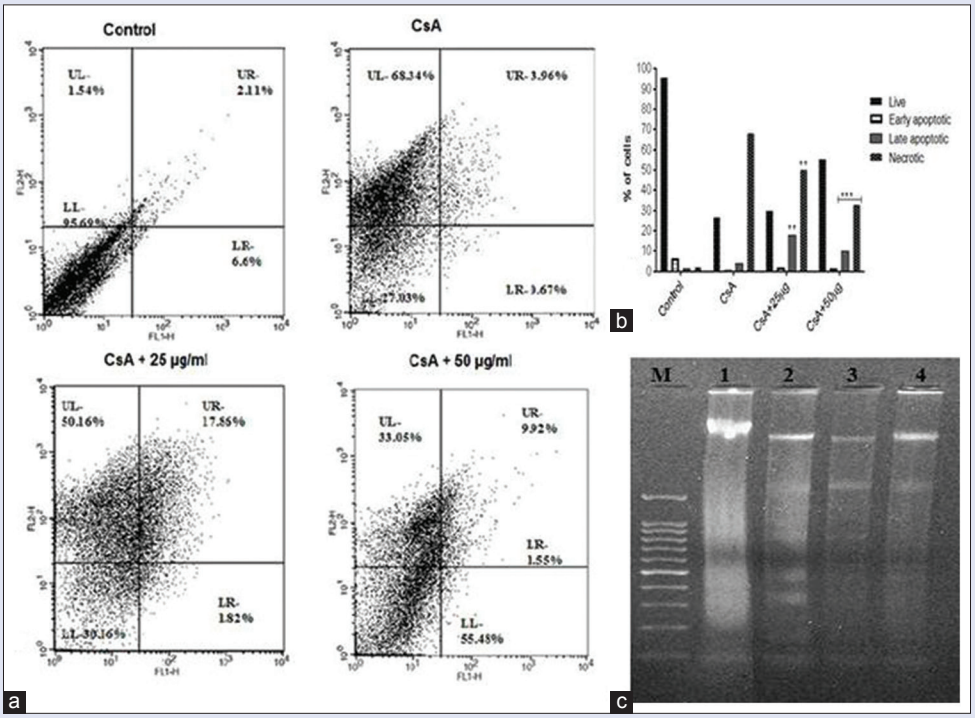

Figure 3: Flow cytometry detection of apoptosis in Madin–Darby Canine Kidney. (a) Cells treated with cyclosporine A and ethyl acetate fraction for 24 h; stained with Annexin V and propidium iodide. (b) Cyclosporine A treatment showed high necrotic cells and necrosis decreased in ethylacetate fraction groups (c) Gel electrophoresis of DNA showed intranucleosomal DNA degradation, displaying dose-dependent reduction in combination of ethyl acetate fraction + cyclosporine A-treated cells (at full page width)

increased in CsA-treated groups [Figure 5a]. EF-treated groups showed diminished ROS levels in MDCK cells. CsA-induced ROS increased the lipid peroxidation which in turn results in the elevated level of malondialdehyde (MDA) ($P < 0.001$) [Figure 5b]. Combined treatment of EF with CsA decreased the MDA level significantly. Nitrite levels were also found to be elevated in CsA-treated groups compared to the co-treatment of EF with CsA ($P < 0.001$) [Figure 5c].

Ethylacetate fraction decreases the collagen production in Madin–Darby canine kidney cell line

The abnormal accumulation of collagen is one of the features in chronic nephrotoxicity. CsA-treated cells showed collagen I synthesis, which was evidenced by staining the cells with direct red. EF reduced collagen I synthesis in a dose-dependent manner ($P < 0.001$) [Figure 6a and b].

Ethylacetate fraction improves the long-term recovery in the cells treated with cyclosporine A

Cell survival was studied through the ability of cells to form colonies after treatment with CsA for 24 h. CsA-treated cells demonstrated low cell survival which improved with distinctly on EF treatment. The cells showed increased growth of cell colonies with increasing dosage ($P < 0.001$) [Figure 7].

Gas chromatography–mass spectrometry analysis of ethylacetate fraction

GC-MS analysis showed the presence of 12 compounds in the EF fraction when compared with the database of NIST [Figure 8]. The analysis showed the presence of phenolic compound, alkaloids, steroid, and nitrogen compounds [Table 2].

DISCUSSION

In our study, we have showed the protective effect of EF of *B. diffusa* against CsA-induced apoptosis in MDCK cells. CsA induces cell death (10 µM) through apoptosis which was evident through cell viability and membrane integrity assay. However, cells exposed to CsA along with EF (25 and 50 µg) significantly decreased the cell death by maintaining the cell membrane integrity, thereby minimizing the LDH release.[21] The CsA-induced cell death was confirmed through Hoechst stain which diffuses through intact membranes of cells and binds to AT rich regions of DNA. Hoechst staining showed apoptosis-induced morphological changes, but these changes were very minimal in the cells treated along with EF which indicates the therapeutic effect of the fraction. Our finding is supported by previous study[22] which elucidated that the bioactive phenolic compounds in *B. diffusa* extract was found to have crucial role in the disease management related with oxidative stress.

In the present study, it is apparent that cells treated with CsA showed noteworthy decrease in DNA synthesis. This was supported by cell cycle analysis of CsA-treated cells with EF which showed a dose-dependent elevation in cell (%) in the sub G_0/G_1 and G_0/G_1 phase with accompanying reduction in the S and G_2/M phase. Healy *et al.*[21] had observed similar CsA-induced blockade in the G_0/G_1 phase of the cell cycle which indicates that DNA is an obligatory target of CsA injury. Following the DNA injury, the damaged cells are either restored by the DNA repair system before proceeding with cell cycle or if the damage is irreversible, then the cells are directed to apoptosis.[23] DNA fragmentation which occurs in response to apoptosis could be due to two apoptotic nucleases; the caspase-activated nucleases and endonuclease G.[24] During apoptotic cell death, phosphatidylserine is translocated to the external surface of the membranes which otherwise faces inside the membrane. In the present study, phosphatidylserine externalization was assessed

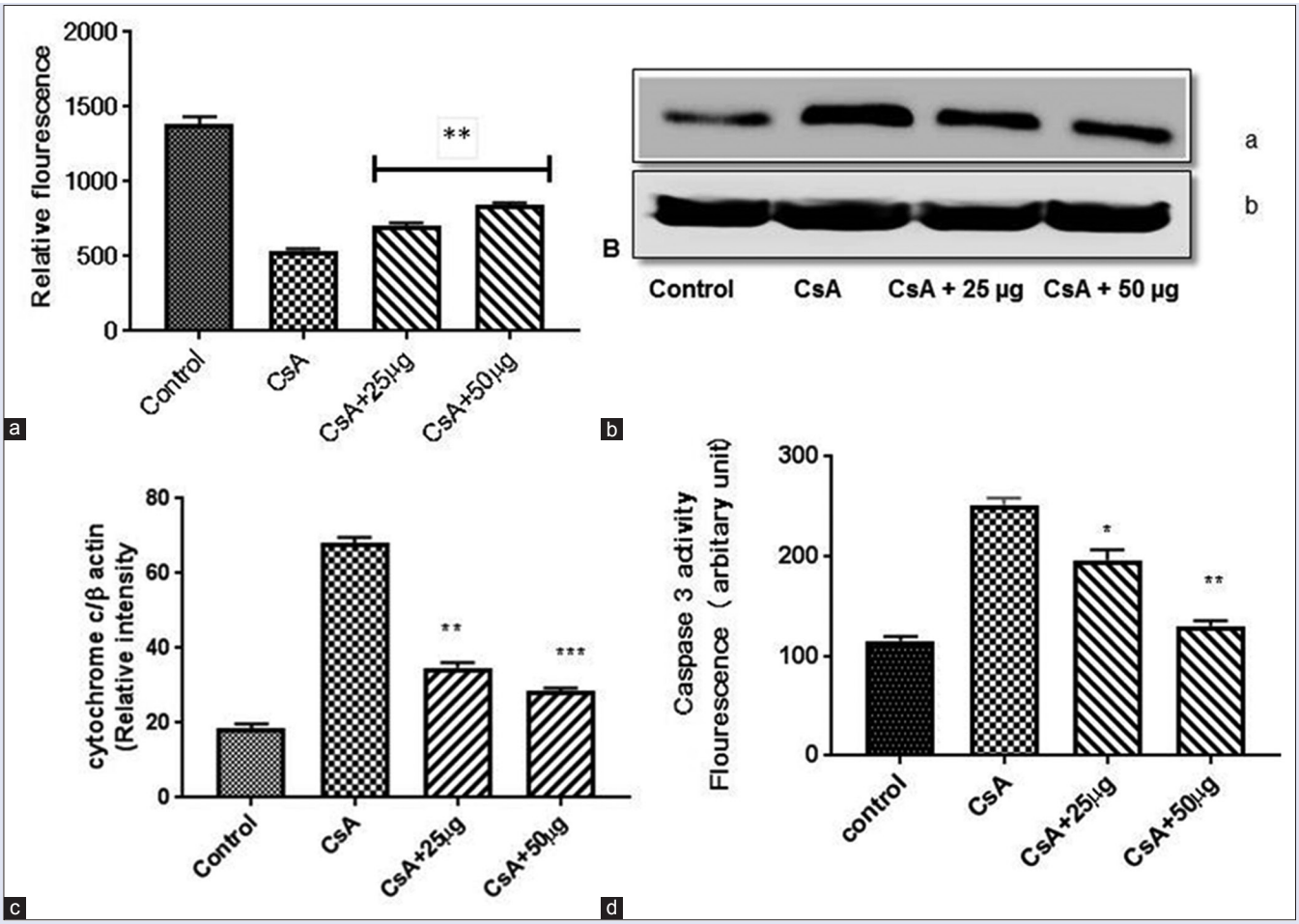

Figure 4: (a) Cells stained with Rhodamine 123 and analyzed by flow cytometry for loss of mitochondrial membrane potential. (b) Immunoblot for cytochrome C (a – cytochrome c; b – β-actin). (c) Relative OD (%) of cytochrome c versus β-actin protein measured against control. (d) ethylacetate fraction suppresses caspase 3 enzymatic activity in cyclosporine A-treated cells (at full page width)

by both Annexin V and PI staining to mark both apoptotic as well as necrotic cells. Cells treated with EF fraction had significantly reduced the percentage of necrosis ($P < 0.0001$).

Oxidative stress ensues by the generation of ROS which defeats the cells' natural antioxidant defense mechanism. Moreover, it is evident that CsA increases ROS production and decreases the antioxidant balance and induces lipid peroxidation.[25] CsA-induced lipid peroxidation results in the increase of MDA which has been suggested to be responsible for oxidative renal damage. Furthermore, CsA-induced free radicals affect the renal tissues by interacting with membrane lipids to produce their peroxidation, thus affecting the cellular structure and function.[26] Nevertheless, EF could salvage this effect through its potential mediated by phenolic substances. As a subsequent event of ROS production, the oxidation of inner mitochondrial membrane (IMM) lipid constituent cardiolipin (CL) also takes place. As a result of which, its stabilization with the mitochondrial complexes and proteins get undermined resulting in disturbance in the membrane electrochemical gradient[2,27] and ends up with opening of mitochondrial permeability transition pores. In addition, it is well-known fact that CL has intimate contact with cytochrome c in the IMM. However, due to the oxidation of CL, cytochrome c is liberated into the intermembrane space.[28] This translocated cytochrome c activates caspase 3, the death sentence of apoptosis. This study also showed that CsA-treated cells present with elevated level of caspase 3 compared to control cells suggesting that the apoptosis was induced through caspase 3-dependent pathway. On the contrary, the cells treated with CsA along with EF has significantly improved the binding of rhodamine ($P < 0.001$), a cationic fluorochrome that binds only to mitochondria of living cells and this decreases cytochrome c as well as caspase 3 release. This indicates that the EF has the potential of preventing the cell death by scavenging the free radicals produced by CsA.

Previous studies have put forward that ROS have a pathogenic role in CsA-induced nephrotoxicity.[2] Similarly, the present study showed that the level of H_2O_2 production is high in CsA-treated cells and on EF treatment the level of H_2O_2 production declined. Consequently, any agent that can reduce nephrotoxicity through scavenging free radicals is of utmost importance in the management of CsA-induced nephrotoxicity. *B. diffusa* with good antioxidant potential may have an important role in attenuating nephrotoxicity. This property could be attributed to the presence of secondary metabolites such as polyphenols (56.68 ± 0.04 µg/mg GAE) and tannin content (191 ± 0.08 µg/mg GAE) in the EF (data not shown). This observation is supported by the previous reports that polyphenols reduce nephrotoxicity by decreasing the oxidative stress through scavenging the free radicals, thereby blocking the further downstream reactions.[29] Polyphenols are gaining much attention for their free-radical scavenging and antioxidant activities because they tend to restrain formation of free radical by chelating transition metal ions and behave as a coantioxidant by increasing the antioxidant activity of other compounds.[27]

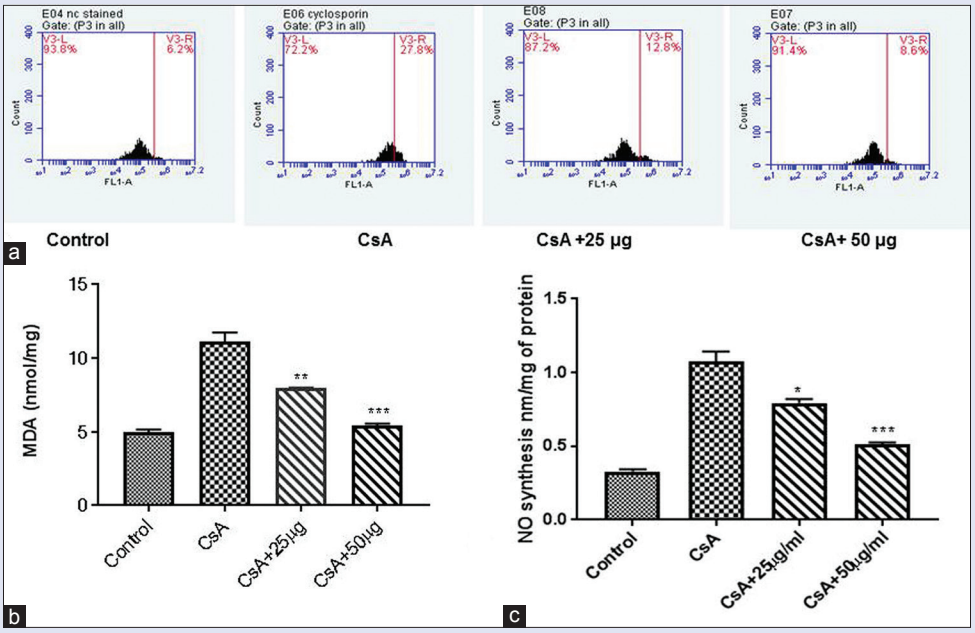

Figure 5: Effect of ethyl acetate fraction on cyclosporine A-induced nephrotoxicity. (a) Cells were loaded with DCFH-DA, harvested, and reactive oxygen species level was measured in flow cytometry. The percentage of reactive oxygen species was increased in cyclosporine A-treated cells, and the combination of cyclosporine A and ethyl acetate fraction reduced the reactive oxygen species level. (b and c) Effect of cyclosporine A and ethyl acetate fraction on malondialdehyde and nitric oxide levels, respectively

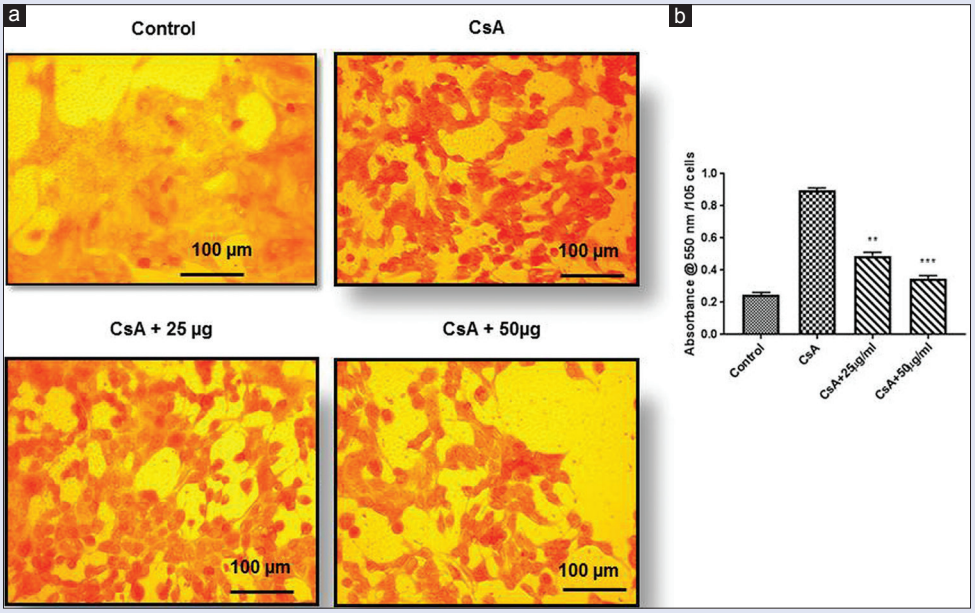

Figure 6: (a) Effect of ethyl acetate fraction on the collagen secretion in cyclosporine A-treated Madin–Darby Canine Kidney cells. After treatment, cells were stained with direct red O to detect the collagen fiber and observed in light microscope. (b) Accumulation of direct red was quantified

Another important consequence of ROS associated with renal toxicity is interstitial fibrosis.[27] Injured tubules occur due to interstitial fibrosis directly or indirectly involved in the synthesis and release of chemokines and cytokines. These cytokines encourage the conscription of macrophages or monocytes and lymphocytes, production and escape of collagen from fibroblast but hinder the loss of interstitial collagen.[30] Cells treated with CsA lead to accumulation of collagen compared to the EF-treated groups. EF-treated cells showed less intensity of the collagen which was dose-dependent, and this effect of the EF could be attributed due to radical scavenging activity.

The next major radical of biochemical importance is nitric oxide (NO) which was significantly found to be elevated in CsA-treated group. CsA-induced increase in peroxynitrite ($ONOO^-$) is due to the interaction between NO and superoxide radicals in the kidney as demonstrated in many studies.[31] This effect is in turn complexed with induction of inducible NO synthase cardinal to the formation of nitrosative stress

Table 2: List compounds present in the EF fraction revealed by the GC-MS analysis

Compound	Nature of the compound	Retention time	Area (%)	Biological property
Carnegine	Alkaloid	4.471	16.35	Molluscicidal activity (Mezghani-Jarraya *et al*., 2009)
N-Benzyl-2-phenethylamine	Alkaloid	4.945	26.35	Acetylcholinesterase inhibition activity (Wang *et al*., 2007)
2-Methoxy-4-vinylphenol	Phenolic compound	5.112	1.95	Anti-cancer activity (Jeong and Jeong, 2010)
Ethyl iso-allocholate	Steroid	5.274	20.6	Anti-microbial, diuretic and anti-inflammatory property (Singariya *et al*., 2012)
Vanillin lactoside	Glycoside	5.382	16.24	-
17-Octadecynoic acid, methyl ester	Ester	5.465	46.61	-
cis-5,8,11,14,17-Eicosapentaenoic acid	polyunsaturated fatty acid	7.269	20.44	Anti hypertensive activity (Hashimoto *et al*., 1998)
1-Heptatriacotanol	Alcoholic compound	7.768	2.55	Antimicrobial activity (Naik *et al*., 2017)
cis-13-Eicosenoic acid	Fatty acid	8.469	6.87	-
Oleic Acid	Fatty acid	12.576	5.5	Anti-oxidant property (Wei *et al*., 2016), hypotensive effect (G. *et al*., 2008)
2-Nonadecanone 2,4-dinitrophenylhydrazine	Ketone compound	17.018	5.75	Anti microbial (Muthulakshmi *et al*., 2012)
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	Volatile organic compound	28.61	33.68	Antimicrobial activity (Venketesh, 2014)

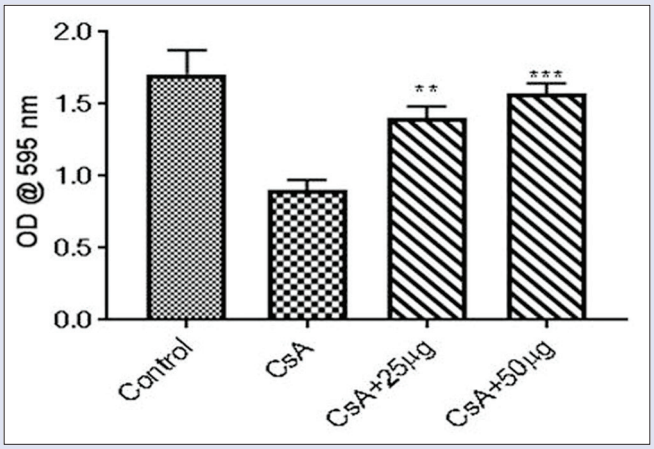

Figure 7: Clonogenic potential of cells after treated with cyclosporine A alone and simultaneous treatment with ethyl acetate fraction and cyclosporine A. Ethyl acetate fraction treatment showed high growth potential dose-dependently. The results represent the average of three experiments

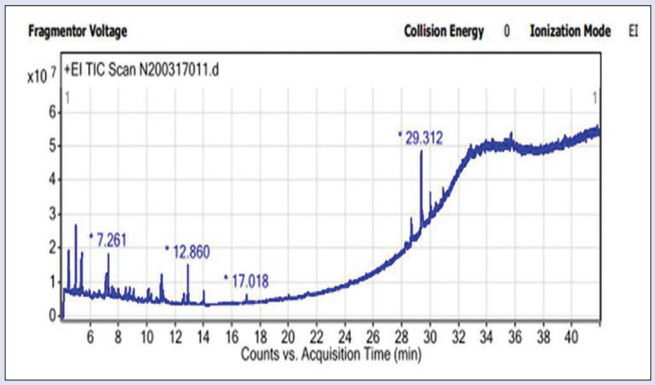

Figure 8: Gas chromatography–mass spectrometry analysis of ethyl acetate fraction of *Boerhavia diffusa* roots reveals the presence of various phytoconstituents. Note: Each bar graph represents the mean ± standard deviation ***P < 0.0001 versus cyclosporine A; **P < 0.001 versus cyclosporine A, *P < 0.5 versus cyclosporine A

which intercede many of the cytotoxic effects.[32] However, cells treated with EF has shown less stress (P < 0.001) compared to the control. This antioxidant property of EF could probably act through two mechanisms. First, with antioxidant potential, it grants an electron to the peroxy radical of the fatty acid, thereby preventing the dissemination step in lipid peroxidation. Second, removal of initiators responsible for radical species formation through quenching the chain-initiating catalyst.[33] The presence of antioxidant compounds such as 2-Methoxy-4-vinylphenol and oleic acid present in the fraction could claim to be responsible for the attenuation of oxidative stress during CsA treatment. Previous studies showed that the anti-oxidant activity soothes oxidative stress and peroxidation reaction induced by CsA and protects kidney tissues.[34]

In addition, CsA hinders the natriuretic and diuretic response to acute volume expansion resulting from RAAS pathway activation.[35] Hypertension and edema are commonly witnessed among the renal and heart transplant patient. To avoid these conditions, treatment with diuretics is recommended.[36,37] Ethyl iso-allocholate, a steroid compound present in the fraction, is also found to possess anti-inflammatory and diuretic activities.[38] The presence of this compound may help in reducing the negative effects of CsA treatment. EF also showed the presence of alkaloids such as Carnegine and N-Benzyl-2-phenethylamine, alcoholic compound, and fatty acids. This is the first report to show the presence of Carnegine and N-Benzyl-2-phenethylamine in the *B. diffusa* plant. Thus, the present study showed the protective effect against CsA-induced nephrotoxicity by the EF of *B. diffusa* by reducing the oxidative stress.

CONCLUSION

Our study data demonstrate that CsA induces cell death through mitochondrial dysfunction and cell cycle arrest. ROS-induced mitochondrial structural damage and hence apoptosis could be rescued by EF of *B. diffusa*. EF protects the mitochondrial deleterious effect through its antioxidant potential by the compounds such as oleic acid and 2-Methoxy-4-vinylphenol. Therefore, this study provides a new comprehensive perspective on this edible medicinal plant in treating drug-induced nephrotoxicity. However, further isolation and characterization of the bioactive compound in EF would be highly appreciated to further explore the therapeutic potential of this exemplary plant.

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Conflicts of interest

There are no conflicts of interest.

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