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In-Vitro Nephroprotective Role of Ethanolic Root Extract of *Boerhaavia Diffusa* Against Cisplatin-Induced Nephrotoxicity

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Abstract

Context and purpose of the study: The present study was carried out to investigate the antioxidant and nephroprotective role of ethanolic root extract of *Boerhaavia diffusa* (ERE).
Results: Antioxidant activity of ERE was studied using DPPH free radical scavenging activity, Nitric oxide scavenging activity and reducing power assay. To study the nephroprotective role of ERE against cisplatin, a porcine renal epithelial cell line (LLC-PK1) was used. Cisplatin increases the apoptotic and necrotic cells and also increases the production of ROS in the treated cells. Co-treatment of ERE decrease the apoptotic and necrotic cells and attenuates the ROS production. This nephroprotective activity could probably be correlated with the phytochemicals like polyphenols (4.5 ±0.02 mg/g), flavonoids (4.2 ±0.08 mg/g) and tannins (6.5 ±0.3 mg/g) present in the extract.
Brief summary and potential implications: These findings suggested that toxicity induced by cisplatin could be reduced by the presence of phytoconstituents in the ERE. This study showed that phytoconstituents present in *B.diffusa* could manage the nephrotoxicity induced by life saving drugs and it can be considered to be given as an adjuvant therapy.
Keywords: Cisplatin, ROS, Oxidative stress, Nephroprotection,B.diffusa root extract.

Introduction

Cisplatin (cis-diamminedichloroplatinum (II) - CDDP) a potent chemotherapeutic drug used in the treatment of various solid organ cancers such as cancers of head, neck, ovary and breast [1]. Even though, cisplatin induced nephrotoxicity has been well recognized for many years, it remains a standard drug because of the less availability of compounds with similar effects. Cisplatin induced cell death is mediated by mitochondrial dysfunction, production of oxidative stress and inflammatory pathways activation like production of chemokines and cytokines [2]. In order to reduce the nephrotoxicity, maneuvers which could reduce the cisplatin without impairing its anti-tumor response is more encouraged. Since ancient times, herbal medications have been used to treat various ailments despite modern medicine has contributed enormously to healthcare. Natural anti-oxidants are very effective in preventing the destructive processes caused by oxidative stress. Based on the substantial evidence on the ROS causing diseases, much attention has been drawn among scientist to exploit the antioxidants incurring the diseases. Hence, the search for novel natural antioxidant of plant origin has been increased. Several studies have been reported the nephroprotective role of anti-oxidants and free radical scavengers of derived forms of plant compounds [3,4]. These plant compounds are known for their anti-oxidant property and they are widely accepted as an adjunct for various diseases along with chemotherapy drugs due to its less or no side effects [5,6].

Boerhaavia diffusa a medicinal plant belonging to the family Nyctaginaceae is used extensively in Ayurveda and Unani practice in the Indian subcontinent. Itpossess a wide array of phytoconstituents like like flavonoids, alkaloids (Punarnavine), steroids, triterpenoids, lipids, lignins, carbohydrates and rotenoids etc [7]. *B.diffusa* has also been used traditionally for its diuretic activity, anti-fibrinolytic, hepatoprotective activity, anti-convulsant, anti-helmintic, cardiotonic, immunosuppressant, anti-viral, anti-inflammatory, anti-diabetic and anti-cancer activities[8]. Nigerian folk medicine uses this plant in the treatment of epilepsy, infertility and dysmenorrhoea [9]. Thus, present study aims at investigating the *in-vitro* anti-oxidant activities and nephro-protective effect of ethanolic root extract (ERE) of *B.diffusa* against cisplatin mediated nephrotoxicity.

Material and Methods

Plant material

The healthy and fresh plant roots were collected from Chennai and Kanchipuram district, India and the plant root was identified and authenticated by (PARC/2010/582) Dr. Jayaraman, Director of Plant Anatomy Research Institute,Chennai, Tamilnadu, India. The collected plant roots were processed, crumbled into coarse powder and stored in the laboratory at the Department of Biomedical Sciences, Sri Ramachandra University, India for reference.

Preparation of ethanolic root extract

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100g of coarse powder material was extracted with 500 ml of absolute ethanol (Merck) by cold percolation method. After three days, the mixture was filtered, concentrated in a water bath around 60- 70º C and stored at -20º C for further use.

Cell Line

The porcine renal epithelial cell line (LLC-PK_1) was obtained from National Centre for Cell Sciences (NCCS), Pune, India, Cells were maintained in *Dulbecco's Modified Eagle's medium* (*DMEM*) with 10% fetal bovine serum (FBS) supplement, 0.5% antibiotics (Sigma) and the cells were maintained in a humidified atmosphere incubated at 37ºC with 5% CO_2 .

Phytochemical Analysis

Qualitative phytochemical screening

The ERE was screened for the presence of active phytoconstituents such as phenols, flavonoids, alkaloids, tannins, saponins, terpenoids qualitatively according to the standard methods[10].

Estimation of Total Polyphenol

ERE was estimated for total phenol content using Folin–Ciocalteau reagent[11]. To 100µl of the extract, 20 µl Folin-Ciocalteau reagent and 50µl 25% sodium carbonate were added. The total volume was made up to 1 ml and kept in dark at room temperature for one hour. The absorbance was measured at 765 nm. The total phenol content was expressed as mg of gallic acid equivalents.

Estimation of Total Flavonoids

The total flavonoid was estimated using aluminium chloride[12]. To 50 µl of the extract, 750 µl of 95 % ethanol, 50 µl aluminium chloride (10%) and 50 µl of potassium acetate (1%) was added and made up to 3 ml with distilled water. The mixture was incubated at room temperature for 30 minutes and the yellow color was measured at 415 nm. The total flavonoid content was expressed as mg of quercitin equivalents.

Estimation of Tannin

To 500 µl of the extract, 0.5 ml Folin's phenol reagent and 5 ml 35% sodium carbonate was added and kept at room temperature for 5 min and the absorbance were measured at 640 nm. The total tannin content of the extract was expressed as mg of gallic acid equivalents [13].

In-Vitro Anti-oxidant activity

In- vitro Free radical scavenging activity

The anti-oxidant property of the plant extract is based on the reduction of 2, 2-Diphenyl-1-picryl hydrazyl (DPPH) in the presence of hydrogen donating compound. To 50 µl of the extract at different concentrations, 150 µl of DPPH was added and incubated in dark for 30 minutes. After incubation, the absorbance was read at 517 nm. Ascorbic acid was used as the standard. IC_{50} value was calculated from the standard equation [14]. The percentage inhibition was calculated from the formula:
% Inhibition= [Absorbance of control-Absorbance of test sample / Absorbance of control] x 100.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured spectrophotometrically using the Griess reagent. To 250 µl of the different concentration of ERE, 625 µl of 5mM sodium nitroprusside was added and incubated for 150 minutes at 25ºC. After incubation, 625 µl Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid) was added and the absorbance was read immediately at 546 nm [15].
(%) NO scavenged = [Absorbance of control-Absorbance of test / Absorbance of control] x 100

Reducing power capacity

This method used to determine the reducing power activity of plant extract by ferric (III) to ferrous (II) transformation. To 1 ml of the different concentration of ERE extract, 2.5 ml of phosphate buffer (0.2 M, pH = 6.6) and 2.5 mL of 1% potassium ferriccyanide was added and incubated in water bath at 50ºC for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added to the mixture and mixed thoroughly. A volume of 2.5 mL of this mixture was then added to 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ and allowed to stand for 10 min. Then, the absorbance of this mixture was measured at 700 nm. Ascorbic acid was used as a standard.[16].

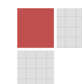
Nephroprotective activity

MTT assay

Nephroprotective activity of the ERE was studied by the MTT assay [17]. Approximately $5x10^3$ cells were seeded in 96 well plate in DMEM medium with 10% FBS in a CO_2 incubator. After the confluency, cells were exposed to 30 µM Cisplatin [18], with and without ERE and incubated for 24 hours. Followed by incubation, the medium was discarded and 20 µl of MTT was added and incubated again for 2 hours. Formazon crystals formed were dissolved using DMSO and measured at 570 nm.

Morphological examination of apoptosis

After simultaneous treatment of cells with Cisplatin and with or without ERE for 24 hours, cells were stained with 10 µg/ml Hoechst



33342 and the changes in morphology was observed under a phase contrast fluorescent microscope (Nikon eclipse Ti) [19] .

Measurement of Necrosis

The extent of necrosis occurred was measured in terms of LDH released from the necrotic cells in the medium [20]. For morphological analysis of necrosis, cells were stained using propidium iodide (PI) stain 10 min, and the cells showing positive PI staining was observed under fluorescence microscopy [21].

ROS Measurement

The intracellular level of ROS was assessed with 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) a cell permeant compound [22]. After 24 hours treatment with Cisplatin, with and without ERE the cells were trypsinized and resuspended in PBS containing 10 μM DCFH-DA and incubate at 37 C for 45 min. Subsequently, the fluorescence was measured at 485 nm excitation and 535 nm emission using a plate reader. All the data are represented as mean ± S.E.M (n=3).

Results and Discussion

Extensive research on plant natural compounds has lead to discovery of potential therapeutic drugs to treat various chronic and infectious diseases [23]. The healing property of medicinal plants is attributed to the presence of several phytoconstituents. The present study aimed to investigate the nephroprotective role of *B. diffusa*against cisplatin induced nephrotoxicity.

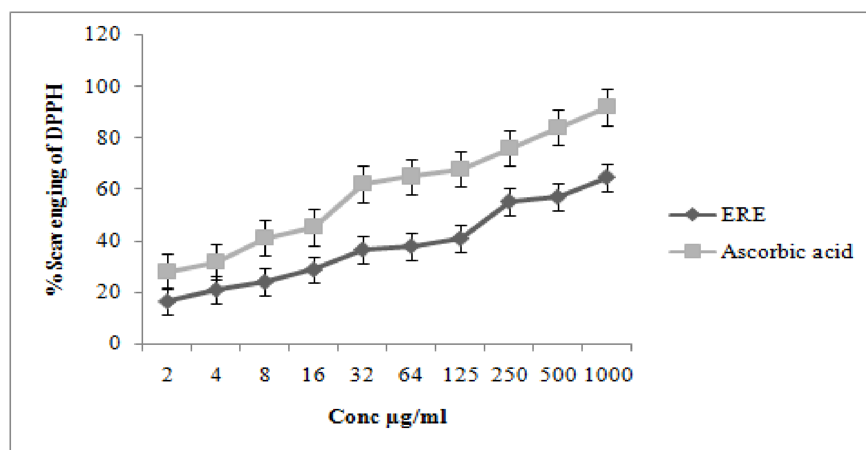
The qualitative phytochemical screening of ERE confirmed the presence of phenols, flavonoids, alkaloids, tannins, terpenoids and reducing sugars but steroid and quinones were found to be absent (Table 1). The quantitative estimation revealed the presence of sustainable amount of polyphenols (4.5±0.07mg/ml), flavonoid (4.2±0.05 mg/ml) and tannin (6.5±0.01mg/ml). These secondary metabolites are highly accountable for their anti-oxidant property and the phenolic compounds are found to be an effective hydrogen donor.

Table 1: Phytoconstituent profile of ERE

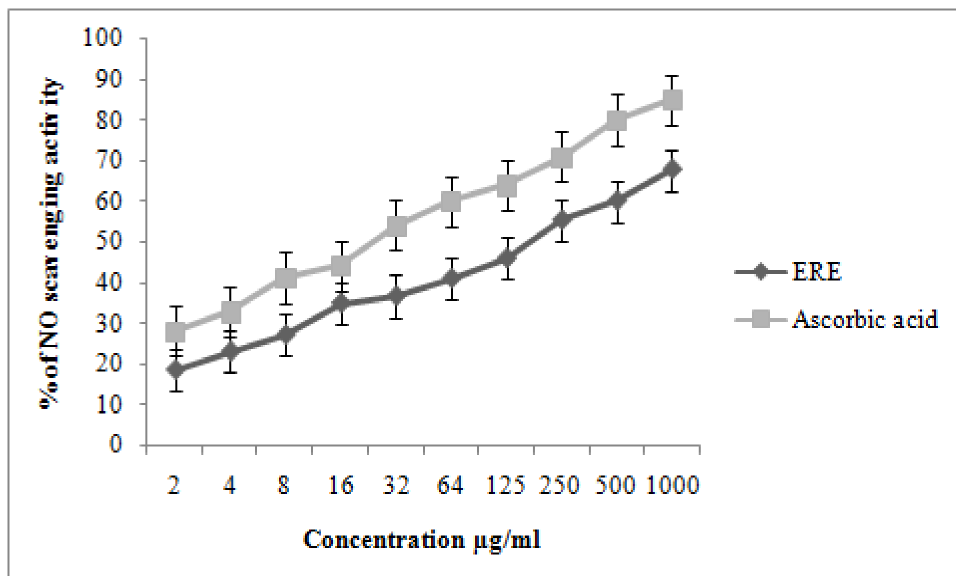
S.No	Phytoconstituents	Root extract
1	Phenols	+++
2	Flavanoids	++
3	Alkaloids	+
4	Steroids	-
5	Tannins	++
6	Terpenoids	++
7	Reducing sugars	++
8	Quinones	-

+ Present - Absent

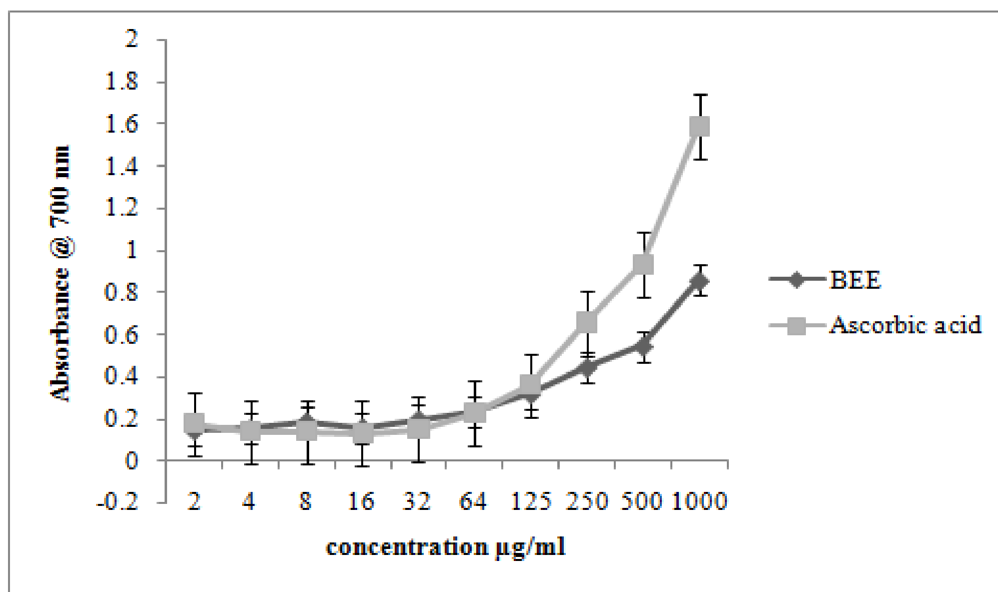
In-vitro anti-oxidant activity of ERE was studied using free radical scavenger (DPPH) and compared with standard ascorbic acid. The IC_{50} value of the ERE was found to be 36.52±0.03 μg/ml and 28 ±0.07 μg/ml for ascorbic acid (Graph 1). The anti-oxidant activity of the ERE was further evaluated by nitric oxide scavenging assay. The nitrite produced by sodium nitroprusside was reduced by ERE in a dose dependent manner. Graph 2 showed the percentage inhibition of nitric oxide production in comparison with standard ascorbic acid. The IC_{50} value of the ERE and ascorbic acid was found to be 30.25±0.006 μg/ml & 26.45 ±0.04μg/ml respectively. Further the potential anti-oxidant property of the plant extract was measured by the reducing power capacity which shows the dose response curve (2-1000 μg/ml) (Graph 3). This result shows that the compounds in ERE are electron donors and it reduces the oxidized intermediates of the lipid peroxidation process thereby reducing oxidative stress [24].



Graph 1: DPPH radical scavenging activity of ERE. Data are presented as the mean ± Standard Mean Error (SME) (n = 3)



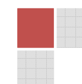
Graph 2: Nitric oxide scavenging activity of ERE. Data are presented as the mean ±SME (n=3)

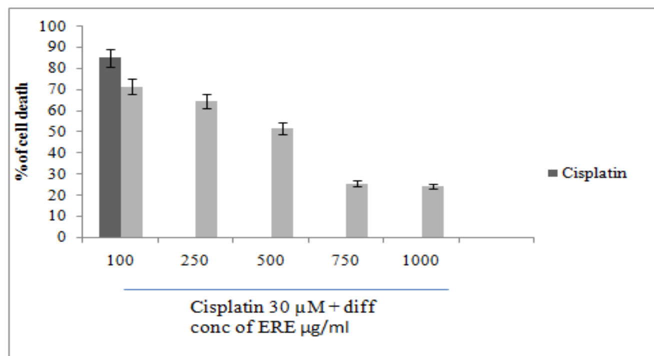


Graph 3: Reducing power activity of ERE. Data are presented as the mean value ± SME (n = 3)

Nephroprotective role of ERE on cisplatin induced nephrotoxicity

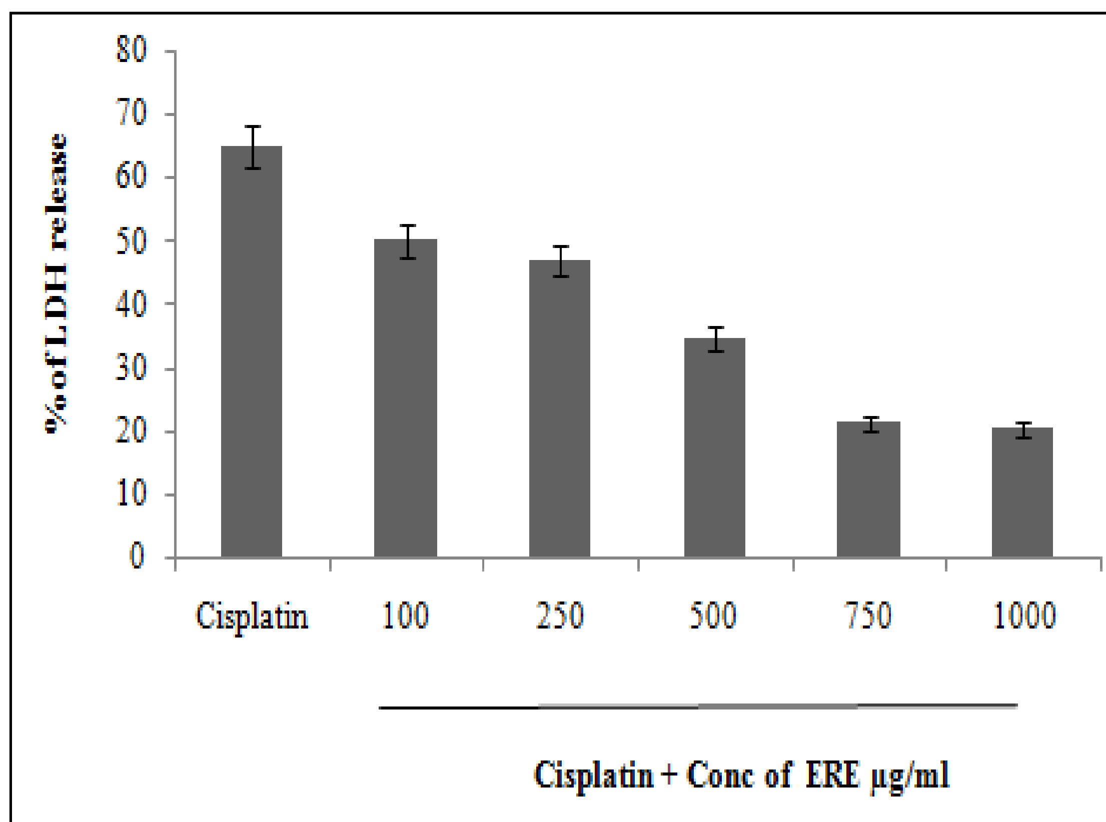
Cisplatin, an antineoplastic drug accumulates in the proximal tubular region of the kidney induces nephrotoxicity and undergoes metabolic activation to a highly reactive thiols and hence its removal is very important for the survival of patient. Since cisplatin accumulates in the proximal tubules of kidney, LLC-PK1 cell line established from porcine kidney epithelial cells was used in this study which mimics the proximal tubule-like features. The protective role of the ERE was studioMTT assay against cisplatin induced nephrotoxicity (Graph 4). When LLC-PK_1 cells were exposed to cisplatin (30 µM) it confers 85% of cell death while cells exposed to cisplatin along with ERE (250 µg/ml & 750 µg/ml), cell death was considerably decreased upto 50% and 25% respectively.





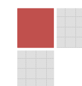
Graph 4: Effect of treatment with ERE on cisplatin-induced cell death in LLC-PK_1 cells. Cells were simultaneously treated with cisplatin and BEE for 24 hours and the cell viability was determined by the ability to reduce MTT. Results presented as mean ± SME from triplicate measurements from at least three independent experiments.

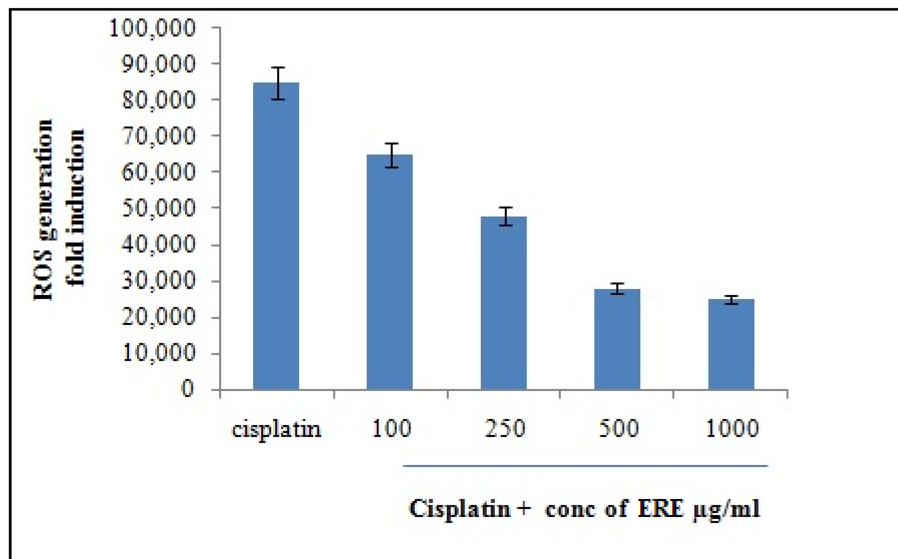
Cisplatin induced nephrotoxicity involves both extrinsic pathway including TNF receptor or FAS and intrinsic mitochondrial and ER stress pathway [25]. Cells treated with cisplatin (30 µM) for 24 hours induces, apoptotic morphological changes (Figure 1) such as cell shrinkage, detachment and nuclear condensation which was confirmed by Hoechst staining (Figure 1b). The cell membrane damage results in Lactate Dehyrogenase (LDH) enzyme release in to the medium and these cells could be bounded with PI due to loss of membrane integrity which is the characteristic feature of necrosis (Graph 5, Figure 1c). However the cells treated along with ERE was found to have less morphological changes that occur due to apoptosis and necrosis.



Graph 5: Effect of ERE on Cisplatin induces the cell membrane damage indicated by LDH release. Data are represented as the mean ± SME (n=3).

Cisplatin is known to accumulate in mitochondria of renal tubular epithelial cells along with ROS. Inspite of continous removal of ROS by mitochondrial antioxidant enzymes (SOD, GPx, CAT and glutathione S-transferase) cisplatin induces the nephrotoxicity primarily by decreasing the activity of antioxidant enzymes and by depleting the intracellular concentration of GSH. Hence in our study ROS accumulated in the renal cells was estimated using DCFH-DA fluorescence assay before and after exposing the cells to ERE (Graph 6). From this study, we observed that ERE treated cells were found to have less ROS accumulation and this property may be attributed to its anti-oxidant property.





Graph 6: Effect of ERE on ROS induced by cisplatin. LLC-Pk1 was incubated with cisplatin with or without ERE for 24 hours. To measure the intracellular peroxide formation DCFH-DA was used as a probe. This compound reacts with the ROS produced in the cells, oxidized and forms the fluorescent product DCF, this fluorescence product was measured as mentioned in the materials & methods. Experiments were performed in triplicates and repeated twice.

FIGURE 1

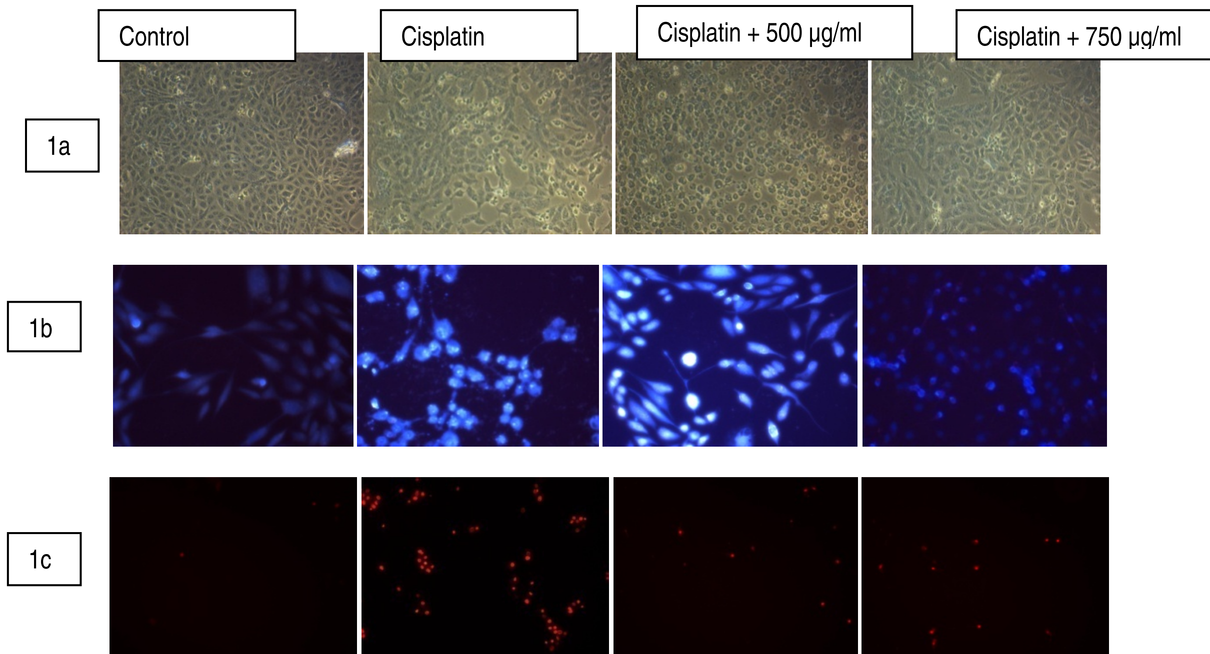
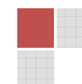


Figure 1: Effect of BEE on cisplatin-induced toxicity. A) Phase contrast images after 24 hours incubated with cisplatin and simultaneously with and without BEE. Control group was incubated without cisplatin and BEE. b) Nuclear staining using Hoechst 33342 shows the apoptotic morphology in the cisplatin treated group. C) PI staining for necrosis measurement.



Various studies have proved that administration of anti-oxidant attenuates the cisplatin induced nephrotoxicity in experimental animal models by reducing the oxidative stress [26]. Dimethylthiourea attenuates the hydroxyl radical production and down regulate the p53 activation thereby it prevents both the oxidative stress and apoptosis [4]. Yet another study evidenced that anti-oxidants such as Vitamin C, Vitamin E and aminoguanidine reduces the MDA levels, and maintains the GSH level in cisplatin treated rats [27]. Similarly in our study antioxidant property of ERE was exploited to reduce the ROS level could be due to the presence of various phytoconstituents in *B.diffusa* which is supported by previous studies where phytoconstituents reduces the cisplatin induced nephrotoxicity. Sibilin, a flavonoid from *Sylibum marianum* was shown to reduce the cisplatin induced nephrotoxicity by reducing the oxidative stress and stabilize the membrane due to its strong anti-oxidant property [28]. Jie Song *et al* (2013) demonstrated that the standardized extract of *Ginkgo biloba* (EGb) containing the flavonoid fraction (24%) has potent biological effects such as free radical scavenging, anti-apoptotic, anti-inflammatory and anti-oxidative activities against cisplatin induced renal cells injury[29]. Thus, in the present study the ERE was shown to attenuate the cisplatin induced nephrotoxicity by reducing the ROS production which may be due to the presence of phytochemical components. But the active principle compound present in ERE needs to be isolated for further study.

Conclusion

These findings suggested that the toxicity induced by cisplatin was considerably reduced during the co-treatment with ERE. This action of ERE could probably be due to the presence of phytoconstituents in the ERE. Further work has to be done to study the compound responsible for nephroprotective role.

Author's contribution

M.K carried out the cell culture study, extraction work and anti-oxidant study. Dr A.S participated in the cell culture study and drafted the manuscript. Dr. H.R contributed to the phytochemical study and performed the statistical analysis. Dr. P.S.R contributed to frame the design of the study and helped in drafting the manuscript.

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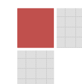

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