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Pharmacological mechanism of indigenous herb *Exacum lawii* on cisplatin instigated toxicity in human embryonic kidney cells (HEK-293)

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ABSTRACT

Background: Exacum lawii C.B. Clarke (Gentianaceae) bitter medicinal herb, has been extensively applied for the treatment of kidney diseases and ailments by Indigenous medical practitioners and local communities in Maharashtra and Karnataka states of India. The pharmacognostical and phytochemical standardization has been performed, but its therapeutic mechanism is still unknown. Swertiamerin, a secoiridoid glycoside established as marker component. The aim of the current research is to investigate the cytoprotective ability and examine targets of action of *Exacum lawii* standardised extract against cisplatin exposed Human embryonic kidney cell line (HEK-293).

Methods: Swertiamerin was isolated from standardised extract using column chromatography and characterized by various analytical techniques. *In-vitro* cellular assay (MTT cytotoxicity assay, iNOS expression, proinflammatory cytokine level, ROS estimation and cell cycle progression, DNA fragmentation assay and morphology of cells) were employed to understand the efficacy and mechanism of action of standardised extract and swertiamerin against cisplatin induced toxicity in HEK-293 cells.

Results: Extract and swertiamerin summed up to be nontoxic to HEK-293 cells and the IC_{50} for cisplatin was 2.261 μg/ml. Molecular docking study showed the binding capacity of swertiamerin for iNOS enzyme (responsible for the nitrosative stress in cells). Various Scientific techniques validates the potential of extract and swertiamerin to reduce the nitrosative stress, oxidative stress, and inflammation in HEK-293 cells significantly ($P < 0.05$) at the dose of 50 μl (2 mg/ml) and 20 μl (0.5 mg/ml) respectively.

Conclusion: This study validates the ethnomedicinal use herb *Exacum lawii* in nephrotoxicity. Swertiamerin as the characteristic bioactive compound might works synergistically with other phytoconstituents to prevent the cellular damage from nitrosative stress, oxidative stress, and inflammation. Therefore, present study emphasizes on the mechanism of action involved in pharmacological activity.

1. Introduction

Cisplatin has been widely used against solid cancer like bladder, cervical, oesophageal, head, neck, ovarian, and testicular cancer (Lebwohl and Canetta, 1998). Anticancer activity of cisplatin depends on formation of DNA intrastrand cross-links with tumor cells (Lippard, 1993). Its use is often associated with nephrotoxicity. Therefore, nephrotoxicity limits the clinical use of cisplatin in 25–30% of patients. However, extensive hydration in patients can partially reduce the extent of kidney injury, but nephrotoxicity remains a main threat (Pabla and Dong, 2008; Arany and Safirstein, 2003). Many studies have established the involvement of reactive oxygen species (ROS) and nitrosative stress for inducing apoptosis in tubular cells or cellular injury of kidney (Chirino et al., 2008; Ju et al., 2014; Ilić et al., 2020).

Abbreviations: HEK-293, Human *embryonic* kidney cells 293; iNOS, Inducible nitric oxide synthase; ROS, Reactive oxygen species; DNA, Deoxyribonucleic acid; MTT, (3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide); DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; RIPA, Radioimmunoprecipitation assay buffer; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF membrane, polyvinylidene difluoride membrane; BCIP-NBT, 5-bromo-4-chloro-3-indolyl-phosphate- nitro blue tetrazolium; TNF-α, Tumor necrosis factor alpha; IL-6, Interleukin 6; ELISA, Enzyme-Linked Immunosorbent Assay; FL1 channel, 525 nm Band Pass Filter; BD FACS, Flow Cytometer from BD Biosciences; TE buffer, Tris-EDTA buffer; FL-2 channel, 575 nm Band Pass Filter; NMR, Nuclear magnetic resonance spectroscopy; ESI-MS, Electrospray ionisation Mass Spectrometry; ANOVA, One way analysis of variance.

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Cisplatin mainly accumulates in the tubular epithelial cells from the S3 segment of the proximal kidney tubules and initiates the cellular toxicity. Several mechanisms have been elucidated for nephrotoxicity which essentially involves the enhanced expression of inducible nitric oxide synthase (iNOS) (Chirino et al., 2008), reactive oxygen species (ROS), proinflammatory cytokines (Ramesh and Reeves, 2002; Faubel, 2007) and direct DNA damage (Leibbrandt et al., 1995). Thus, the inhibition of oxidative stress and inflammatory cytokines along with the cell cycle inhibition, that can be the important target for preventing renal toxicity, which allows time and juncture for damaged DNA to get repaired and complete the regeneration and replacement process.

Exacum lawii C.B. Clarke in Hook species of genus Exacum (family gentianaceae), is small annual herb (Clarke 1885). It is endemic to Jarandeshwar hill of Satara district, Maharashtra and Western ghat of Karnataka, India. The common English name is Law's Persian violet. It is locally known as Lahan chirayata in Maharashtra, Manali in Malayalam, Marukozhunthu in Tamil. The whole herb possesses medicinal property and has been used as traditionally for the treatment of kidney problems and eye diseases (Gamble, 1923; Kirtikar and Basu, 1935; Chopra et al., 1956; Henry et al., 1987). The medicinal uses of *Exacum lawii* was also confirmed from the traditional medical practitioners of local areas near Mahabaleshwar in Satara district of Maharashtra (Damaji and Khandekar, 2011; Vinayaka et al., 2016). The quality control standardization of *Exacum lawii* and DNA finger- printing profile has been performed in our previous study according to WHO guidelines.

Plants belonging to the family Gentianaceae are known for their bitter taste, which is supported by many compounds, including swertiamarin. Swertiamarin (secoiridoid glycoside) has received extensive interest due to its abundant occurrence in medicinal plants and has wide range of molecular targets for anti-inflammatory and antioxidant activity (Muhamad et al., 2021). The phytochemical standardization of ELE confirms the presence of swertiamerin through TLC and HPLC. Consequently, Swertiamerin was considered as a marker compound and isolated from the standardised extract.

Ethanolic extract of *Exacum lawii* (ELE) was also found to be rich in various phytoconstituents such as flavonoids, phenols, bitter secoiridoids and xanthons (Sharma and Hemalatha, 2017). ELE have the nephroprotective action in cisplatin treated experimental rats (Sharma et al., 2017). Therefore, it seeks attention to explore its effect and mechanism of action against cisplatin treated human embryonic kidney cells (HEK-293).

Initially its action on iNOS enzyme, which is responsible for nitrosative stress was diagnosed by virtual investigation. The molecular docking software AutoDock 4.2 was used for studying its binding interaction within the active site of iNOS (PDB ID: 3NQS).

Further, the study involves the western blot analysis for iNOS expression, estimation of proinflammatory cytokines and ROS level, cell cycle analysis, DNA fragmentation assay and morphological analysis of treated HEK-293 cells. It helps us to acquire the insight to its molecular mechanism for pharmacological action.

2. Material and method

2.1. Isolation and characterization of swertiamerin from standardised Exacum lawii extract

The herbs were collected in the month of August-October from Mahabaleshwar, Maharashtra, India. The herb was identified and authenticated by Dr. N. M. Dongarwar, Assistant Professor, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, India. Voucher specimen (Cog/EL/2014–15) was deposited for future reference in Pharmacognosy laboratory of Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi, India. Pharmacognostical standardization was performed as per WHO guidelines (World Health Organisation, 2002). and RAPD DNA fingerprinting profile was established with Eurofins Analytical Services India. DNA was extracted using commercial kit (ThermoFisher Scientific), Chloroplast and nuclear sequence regions were PCR amplified from isolated DNA using gene specific primer sets (rbcLaF & rbcLarR and MatK_2.1F & MatK_5R) analyzed by DNA sequencing followed by data base search in the nucleotide data base of the US National Center for the Biotechnology Information (NCBI) (Sharma et al., 2017).

The dried plant powder was defatted with petroleum ether extracted in ethanol. The obtained ethanolic extract was filtered and concentrated. The obtained concentrated extract was treated with cold diethyl ether and the precipitate obtained was triturated with silica gel (60–120 mesh, E. Merck, Germany) and loaded on a column and eluted with mixture of petroleum ether and ethyl acetate (1–20%). The different elutes which showed similar spots in TLC by eluting with a mixture ethyl acetate containing increasing ratio of methanol (0–10%) were collected and pooled together. Finally, the eluates which showed single spot with solvent system of ethyl acetate: methanol: water (7.7: 1.5: 0.5) were combined and concentrated to dryness in vacuum. The compound was confirmed by Co-TLC with standard swertiamarin (Natural remedies Pvt Ltd). Recrystallization and purification were done by dissolving the residue in methanol (Vishwakarma et al., 2004)

The isolated compound was identified to be swertiamerin by measuring its melting point using open capillary tubes on a Stuart melting point apparatus (SMP-10). UV absorption spectrum (Shimadzu-1700, Double beam), Infrared absorption spectrum (FT-IR spectrophotometer, Shimadzu model 8400, Tokyo) and HPLC standardization were also recorded. The structure of swertiamarin was elucidated by ^{1}H NMR (300 MHz and 500 MHz) and ^{13}C NMR (75 MHz and 125 MHz) and data were recorded on a bruker advance HD NMR spectrometer equipped with 5 mm multi nuclear BBFO probe with Z- gradient facility in DMSO-d6 using TMS as an internal standard. ESI-MS data was taken using LCMS (Thermo Fisher Scietific) with ion trap ESI-MASS detector. The data obtained was analysed and compared with literature (Rai et al., 1966; NCBI database)

2.2. Molecular docking study for isolated swertiamerin as marker compound

2.2.1. Protein structure and ligand preparation

The crystal structure of NOS heme domain (PDB ID: 3NQS) was retrieved from the RCSB protein data bank (PDB) (http://www.rcsb.org). The protein has two polypeptides and is co-crystallized with the ligand and heme. Water molecules and ligands were removed, and hydrogen atoms were added to the amino acid residues. The protein was optimized and energy minimised. Virtual screening and molecular docking study of swertiamerin as selective inhibitor of inducible nitric oxide synthase enzyme was performed. The docking study was done using AutoDock 4.2 package software to investigate the affinity of swertiamarin to the binding pocket of iNOS (Petchiammal et al., 2011; Morris et al., 2009). Swertiamarin was downloaded as mol2 file from zinc database and the energies of compound was minimised. The compound was converted into the pdb format using open Babel 2.3.1 software. During the docking, the grid dimensions were 50 × 48 × 54 Å with points separated by 0.375 Å. The X, Y and Z coordinates were specified as 124.559, 113.056 and 35.429, respectively. The Lamarckian genetic algorithm was applied for energy minimization using default parameters. The number of docking runs was 10, the population in the genetic algorithm was 150, the number of energy evaluations was 2500,000 and the maximum number of iterations was 27,000. The default settings were used for all other parameters. At the end of docking, ligands with the most favourable free energy of binding were selected as the resultant complex structures. All calculations were carried out on PC based machines running Windows 7, 32 bits as operating system. The resultant structure files were analysed using Discovery Studio Visualizer 3.1 obtained from (www.accelerys.com).

2.3. Screening of standardized extract for nephroprotective activity in HEK-293

2.3.1. MTT cytotoxicity assay

Equal number (1000 cells) of HEK 293 cells were seeded in 96-well culture plates and incubated in a 5% CO_2 incubator at 37 °C for 48 hour and cells were allowed to adhere and achieve 70–80% confluency. Cell survival was assayed by colorimetric methyl thiazolyl diphenyl-tetrazolium bromide assay (MTT, Himedia, India). The cells were incubated with different concentrations of cisplatin (Cytoplatin-10, Cipla) (1 μg/ml to 7 μg/ml), ELE (2 mg/ml to 14 mg/ml) and swertiamerin (0.5 mg/ml to 2 mg/ml) in different wells of each row having freshly prepared media. After 48 hour of drug treatment, 20 μl of MTT solution (6 mg/ml of PBS) was added to each well of a 96-well plate and the plates were incubated for 3 h at 37 °C. Viable cells allow to convert the yellow-coloured MTT into dark-blue formazan crystals. The total number of formazan crystals formed was solubilized by adding 20 μl of DMSO and was examined by measuring the absorbance at 570 nm using a microplate reader (BioRad). The assay was carried out in triplicate. The IC_{50} value of Cisplatin was obtained by plotting the relative cell viability percentage versus the concentration of the test compounds. (Singh et al., 2016)

2.3.2. HEK-293 cell culture maintenance and drug treatment

Human Embryonic Kidney cell line, HEK-293 obtained from the National centre for Cell Sciences (Pune), Maharashtra, India. The HEK-293 cells were grown and maintained in Dulbecco's Modified Eagle Medium, supplemented with 10% heat inactivated foetal bovine serum and 1% penicillin- streptomycin antibiotic combination (Invitrogen Life Technologies, Rockville, USA) at 37 °C in a humidified atmosphere with 5% CO_2. Trypsin was obtained from HiMedia Laboratories (Mumbai, India). (Singh et al., 2016)

For each experiment (Western Blot Analysis, Pro-inflammatory cytokine, ROS estimation, Cell cycle analysis, DNA fragmentation assay, Morphological analysis) equal number of HEK- 293 cells (1.0×10^5) was seeded for 24 h in a 6-well culture plate and labelled. Control (no drug treatment), toxic control (3 μl of cisplatin, 1 μg/ml) after 24 h of seeding the cells, extract treated (50 μl of extract (2 mg/ml) along with 3 μl of cisplatin (1 μg/ml)), swertiamerin treated (20 μl of swertiamerin (0.5 mg/ml) along with 3 μl of cisplatin (1 μg/ml)). The HEK-293 cells from each treatment group were harvested by trypsinization for further experiments after 48 h of drug treatment.

2.3.3. Expression of iNOS by western blot analysis

Total cellular protein was extracted from HEK-293 cells of treated and control group. The cells were harvested and centrifuged with 800 μl PBS at 3000 rpm for 6 min, RIPA buffer (Sigma, USA) and protease inhibitor cocktail phenylmethane sulfonyl fluoride (Sigma, USA) was added to cell pellet obtained. The whole cell lysates were vigorously pipetted up-down few times and vortexed. Finally, lysate was centrifuged at 4 °C for 10 min at 12,000 rpm and stored at −80 °C. Equal number of proteins (50 μg) were resolved by 8% SDS-PAGE and electrophoresis was performed in tris-glycine buffer with marker protein, band were transferred in transfer buffer to PVDF membrane (Millipore, USA) followed by blocking with 5% skimmed milk and anti-GAPDH antibody (sc-32,233) antibody was added for overnight at 4 °C. The membrane was washed thrice with TBST. Anti-iNOS (sc-7271) specific antibodies were added and membrane was washed with after 3 h. Finally, immunodetection was performed with dye BCIP-NBT (Sigma, USA) (Ben et al., 2011; Smith and Titheradge, 1998).

2.3.4. Estimation of pro-inflammatory cytokine

Proinflammatory cytokines levels in protein sample isolated from treated HEK-293 cells had been investigated by measuring levels of TNF-α and IL-6 using standard sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit (Komabiotech, Korea) according to the manufacturer's instruction. Standard and detection antibodies provided were reconstituted in sterile water. Serial dilutions of standard and samples were prepared. Selected wells in microplate were washed with washing solution. A volume of 100 ml of samples and standard were added to wells followed by addition of diluted detection antibody after incubation. Color was developed with the addition of color development enzyme and color development solution. Finally, absorbance was measured using microplate reader (BioTek Instruments Inc., USA).

2.3.5. Estimation of ROS level by flow-cytometry

Briefly, HEK-293 cells were harvested the cells were collected by trypsinization, centrifuged at 3000 rpm for 10 min, and washed twice with chilled 1 × PBS (pH 7.4), followed by centrifugation at 2000 rpm for 5 min. The cells were re-suspended in 1 × PBS and 20 μM of 2′, 7′-Dichlorofluorescein diacetate (DCFDA, Sigma) was added and incubated in dark for 15 min at 37 °C. Fluorescence intensity was acquired at FL1 channel with BD FACS calibur (BD Biosciences). 10,000 events were taken, and data was analysed in Cell Quest Pro-software (Becton Dickinson, Franklin Lakes, New Jersey, USA).

2.3.6. Cell cycle analysis of HEK-293 cells

The harvested HEK-293 cells from treated and control group were centrifuged at 3000 rpm for 10 min and washed twice with chilled 1 × PBS (pH 7.4), followed by centrifugation for 5 min at 2000 rpm. The pellet was suspended and fixed in chilled ethanol (70%) for 30 min at 4 °C, again centrifugation at 4000 rpm for 15 min, and suspended in 1 × PBS. The RNase-A (5 μl) was added and incubated (10 mg/ml) for 30 min at 37 °C, followed by staining with 10 μl Propidium iodide (Sigma Aldrich, Bengaluru) (1 mg/ml) for 30 min. The cell cycle distributions for each sample were analysed using Cell Quest Pro-software, fluorescence intensity was acquired at FL-2 channel with BD FACS calibur (BD Biosciences) (Singh et al., 2016).

2.3.7. Qualitative DNA fragmentation assay

HEK-293 cells from treated and control group were harvested and lysed in 50 μl of lysis buffer (50 mmol/liter of Tris–HCl (pH 8.0) and 0.5% SDS) and incubated for 30 min at 37 °C in water bath. To ensure uniform mixing of cells with lysis buffer, the cell pellet was stirred with a wide-bore pipette tip followed by the addition of 2 μl of 10 mg/ml DNAase free RNase (Thermo scientific™). The mixture was incubated for 2 h at 37 °C. Samples were further added with 5 μl of Proteinase K (Thermo scientific™) solution and incubated at 50 °C for overnight. The precipitated DNA was dissolved in a 20 μl of TE buffer (1X) and quantified spectrophotometrically (Kasibhatla et al., 2006). An equal concentration (5 μl) of DNA was resolved on 1% agarose gel stained with ethidium bromide. Gel was observed under UV light, followed with documentation using the Alpha Innotech system (San Leandro, California, USA).

2.3.8. Morphological analysis

The HEK-293 was grown in a six well culture plate at a density of 1×10^5 cells/well plate for 24 h under standard culture conditions, the cells were further incubated for 48 h after drug treatment. Then, morphological changes in the cells were examined using an inverted microscope) with fluorescent lamps and digital cameras (Olympus BX53F; Olympus, Tokyo, Japan). The data were acquired and analysed using Cell Sens software (Olympus) (Singh et al., 2016).

2.4. Statistical analysis

Analysis of variance had done by one way analysis of variance (ANOVA) followed by Newman–Keuls Multiple Comparison test for determining the statistical significance between different groups. A difference in the mean values of $p < 0.05$ had considered to be statistically significant. Graph Pad Prism 5.0 software (Graph Pad software, Inc., La Jolla, CA) had been used for all statistical analysis (Li et al.,

2017).

3. Results

3.1. Isolation and characterization of swertiamerin from standardised Exacum lawii extract

The referential information to develop a monograph for quality control standardization of *Exacum lawii* was reported. The isolated swertiamerin (R_f value 0.57) was standardized and characterized by various analytical techniques. The melting point was found to be same as swertiamerin that is 113̊ C to 114̊ C. UV absorption maxima (λ max) was found to be 238 nm in methanol. The best base line separation (peak purity > 95%) for swertiamarin was achieved at 238 nm with HPLC analysis. Structure of isolated swertiamerin was elucidated by infrared spectrum with characteristic peak 3439.19 cm^{-1} to 3404.47 cm^{-1} for –OH stretching, 2922.25 cm^{-1} for CH_2 stretching, 1695.49 cm^{-1} for C = O stretching, 1616.40 cm^{-1} for CH═CH stretching, 1411.94 cm^{-1}C=C binding and 1282.71 cm^{-1} for C—O stretching, Electrospray ionization-mass spectrometry (ESI-MS) analysis of swertiamarin showed that the pseudo-molecular ion peaks 397.50 [*M*+Na+], 771.27 [2M+Na+] which confirm the molecular weight of the compound is 374.34. (Rai et al., 1966; National Library of Medicine US, 2004 PubChem database).

3.2. Molecular docking study for isolated swertiamerin

The isolated swertiamerin was docked successfully into the catalytic site iNOS using the automated molecular docking program AutoDock 4.2 as per the protocol mentioned above. The compound swertiamerin showed binding energy −6.38 kcal/mol. Inhibition constant obtained was found to be 21.17 mM. It was properly positioned in the enzyme cleft visual inspection of binding modes of inhibitor within the active site of iNOS. It was made to identify the binding orientation and possible interactions within the active site of the iNOS. Information regarding binding orientation and potential- inhibitor enzyme interaction was obtained. The ligand showed π-π interaction with trp 188 and phe 36. The binding of compound is also stabilised by π- sigma interaction with gly 196 (Figs. 1-3) (Table 1).

3.3. Screening of standardized extract for nephroprotective activity in HEK-293

3.3.1. MTT cytotoxicity assay

The MTT cytotoxicity assay was performed to assay the cytotoxic effects of Cisplatin, extract and swertiamerin against HEK-293 cell line and to select the dose for further study. Relative cell viability for HEK-293 cell lines was measured at seven different concentrations for each cisplatin extract and swertiamerin. The obtained growth curve for cisplatin showed that cisplatin causes toxicity, reduction in cell viability was dose dependent. The inhibitory concentration 50% (IC_{50}) value of cisplatin was found to be 2.261 μg/ml. Extract and swertiamerin

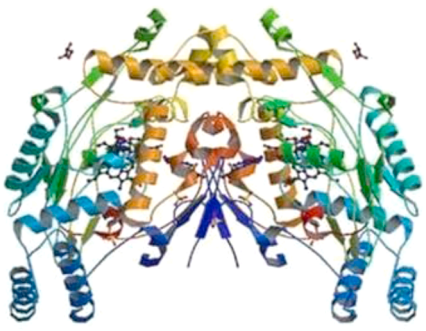

Fig. 1. Swertiamerin −3NQS docking showing different bonds.

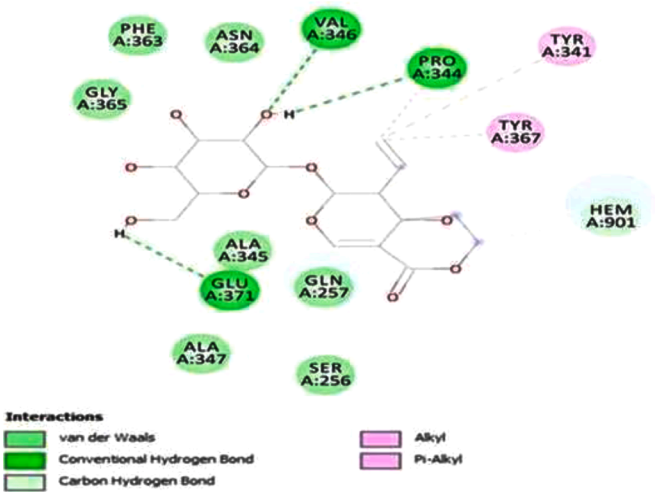

Fig. 2. Swertiamerin −3NQS docking showing different predicted bonding interactions.

treatment did not affect relative cell viability up to the dose of 14 mg/ml and 2 mg/ml respectively (Fig. 4). Initial concentrations of cisplatin, extract and swertiamerin were taken for further molecular study.

3.3.2. Expression of iNOS by western blot analysis

Cisplatin, 3 μl (1 μg/ml) treatment enhanced the expression of iNOS enzyme in HEK-293 cells. Western-blot analysis validated the significant downregulation of iNOS expression by ELE, 50 μl (2 mg/ml) than swertiamerin, 20 μl (0.5 mg/ml) treatment in cisplatin administered HEK-293. Blot obtained was compared to the respective untreated cells (Fig. 5).

3.3.3. Estimation of pro-inflammatory cytokine

Cisplatin, 3 μl (1 μg/ml) administration in HEK-293 results in the upregulation of proinflammatory cytokines level (IL-1β and TNF-α). The treatment with ELE, 50 μl (2 mg/ml) was found to be more effective in lowering the level of proinflammatory cytokines in HEK-293 to normal level than swertiamerin, 20 μl (0.5 mg/ml) (Fig. 6).

3.3.4. Estimation of ROS level by flow-cytometry

The Flow cytometric measurement was performed to verify the mitochondria generated ROS in treated and untreated HEK-293 cells. Data for Geometrical mean (Geo Mean ± SD) values of fluorescence intensities for each group from two independent experiments are overlay histogram and bar graph. Geometrical mean for untreated cells was 57.63, for cisplatin, 3 μl (1 μg/ml) treated cells was found to be 124.08. The ELE, 50 μl (2 mg/ml) reduces the ROS level in HEK-293 significantly ($P < 0.05$) in cisplatin treated HEK-293 cell line with Geo metrical value 77.36 and for swertiamerin, 20 μl (0.5 mg/ml) treatment Geo metrical mean was 89.74 (Fig. 7-8).

3.3.5. Cell cycle analysis of HEK-293 cells

Flow cytometric analysis determines the distribution of cells in various stages of the cell cycle. Result showed that, after cisplatin administration the cells enter cell cycle and the percentage of G2/M phase in cisplatin treated HEK-293 was found to increase up to 61.29% from 27.93% of control. Treatment with ELE and swertiamerin released the cells from the G2/M phase and increased number of cells in G1 phase from 6.58% in cisplatin to 18.53% in ELE and 15.53% in swertiamerin treated cells. The different phases of the cell cycle and the percentages of sub-G1, G1, S, and G2/ M phase cells are presented graphically (Fig. 9).

3.3.6. DNA fragmentation assay

DNA Fragments were observed in cisplatin treated cells against 1 kb

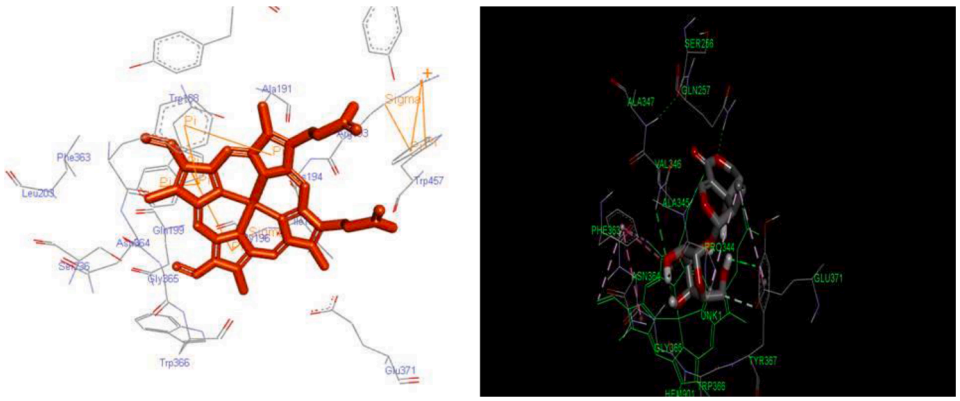

Fig. 3. Swertiamarin-3NQS docking shows different predicted bonding interactions.

Table 1
Interactions obtained after molecular docking study.

S. No.	Interaction type	Protein residue	Ligand	Distance (Å)
1	**H-bond**	Hem901	CH	2.92
2.	**H-bond**	Val-346:NH	OH	2.74
3.	**H-bond**	Glu-371: CO	OH	2.37
4.	**H-bond**	Pro-344:	OH	2.94
5.	**Alkyl interaction**	Pro-344:	CO	5.44
6.	**Alkyl interaction**	Tyr367	CO	5.09

ladder, ELE and swertiamerin prevent the DNA damage when compared with untreated cells (Fig. 10). No fragments were formed in the DNA of HEK-293 cells treated with ELE and swertiamerin after cisplatin treatment.

3.3.7. *Morphological analysis*

As shown in (Fig. 11) untreated HEK-293 cells were adherent, flattened thin elongated with 80% confluency. Cisplatin administered cells were floating and less adherents, with more apoptotic bodies, cytoplasmic vacuoles, shrinking cells, shape becomes more spherical, formation of blebs on the surface. Treatment with ELE showed better defense than swertiamerin alone and helps the cells to conserve their shape and cellular structure.

4. Discussion

The use of traditional medicines and nutraceuticals or phytonutrients continues to embellish across the world for treatment of various health challenges in different national healthcare settings. It contributes to sustained use and conservation of this ethnomedicinal plant (Bandaranayake, 2006; Bodeker et al., 2005).

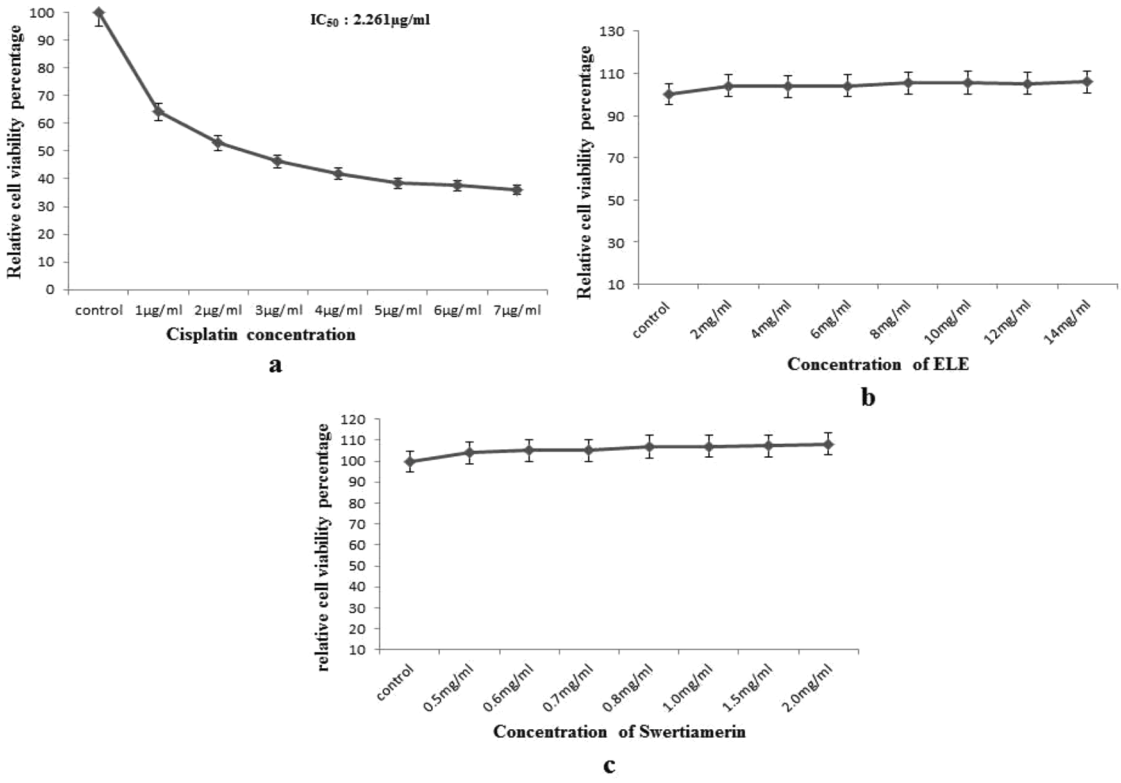

Fig. 4. Effect of cisplatin, ELE and swertiamerin on relative cell viability by colorimetric methyl thiazolyl diphenyl-tetrazolium bromide assay (MTT assay). Obtained growth curve showing a. Cisplatin is toxic to cells and lowers the cell viability in a dose dependent manner (IC_{50}) value of cisplatin is 2.261 µg/ml. b. ELE does not affect the relative cell viability. c. Swertiamerin does not affect the relative cell viability.

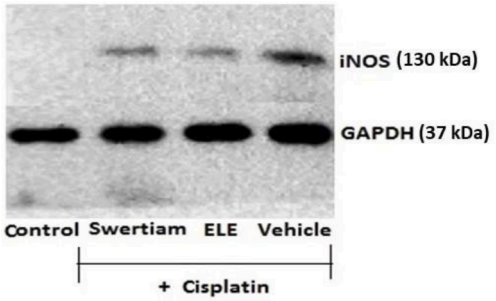

Fig. 5. Blot showing cisplatin induced marked increases in protein iNOS expression and attenuation of cisplatin-induced renal overexpression of iNOS by ELE and swertiamerin treatment. The blots were stripped and reprobed for GAPDH protein as a loading control.

In accordance with the results obtained, Swertiamerin was successfully isolated from whole plant of *Exacum lawii* in the present study and confirmed from the public database. The melting point and UV absorption maxima of swertiamerin obtained was compared with literature found to be 113 °C to 114 °C and 238 nm respectively. FTIR spectra was recorded and compared with literature. The structure was also confirmed by H-1 NMR, C-13 NMR and ESI-MS data. Electrospray ionization-mass spectrometry (ESI-MS) analysis of swertiamarin showed that the pseudo-molecular ion peaks 397.50 [M+Na^+], 771.27 [2M+Na^+] which confirm the molecular weight of the compound is 374.34 and mass spectrum also confirmed the molecular formula as $C_{16}H_{22}O_{10}$.

Cisplatin (cisplatinum or *cis*-diamminedichloroplatinum (II)) is one of the most effective and widely used chemotherapeutic agents. Nephrotoxicity its major side effect was reported in the initial clinical trials of cisplatin chemotherapy (Hill and Speer., 1982). The inhibitory concentration 50% (IC_{50}) value of cisplatin was found to be 2.261 μg/ml on HEK-293 cells. The pathogenic events involving oxidative stress (implicate reactive oxygen species), nitrosative stress (implicate reactive nitrogen species) and inflammation leads to DNA damage in renal cells after cisplatin accumulation (Yan et al., 2016). Thus, the defensive mechanism was evaluated by performing experiments involving measurement of iNOS level, proinflammatory cytokine levels, ROS, and further DNA damage.

The MTT cytotoxicity assay, results manifest that cisplatin treatment decimates 50% of HEK-293 cells at the dose of 2.261 μg/ml. ELE and swertiamerin treatment were found to be safe and nontoxic to HEK-293 up to the higher dose of 14.0 mg/ml and 2.0 mg/ml respectively.

Pathogenesis of nephrotoxicity primarily involves oxidative stress and nitrosative stress (implicate reactive nitrogen species) in kidney cells. Reactive nitrogen species like $OONO^-$, NO_2Cl, NO_{2° and peroxynitrous acid originates from reaction of mitochondrial superoxide with nitric oxide free radicals (NO°), these free radicals are synthesised from enzyme family Nitric oxide synthase (NOS) localised in kidney. The enzyme iNOS (inducible nitric oxide synthase) is usually up regulated in response to lipopolysaccharides, cytokines, and oxidative stress (Fleury et al., 2002). Eventually, an increase in iNOS mRNA level has been seen in cisplatin administered kidney (Chirino, 2008). Thus, the isolated swertiamerin from the extract was investigated virtually for their binding interactions within the active site of iNOS (PDB ID: 3NQS) using the molecular docking software AutoDock4.2 and the docked conformations showed the binding energy of −6.38 Kcal/mol. Swertiamerin exhibited inhibition constant with a Ki of 21.17 mM.

The results obtained from western blot analysis for iNOS expression indicates cisplatin administration in HEK-293 cells causes enhanced iNOS expression which further causes nitrosative stress. The extract and swertiamerin treatment in cisplatin prior treated cells produces lower expression of enzyme iNOS.

Inflammation also plays a critical role in cisplatin induced toxicity with involvement of leukocytes, chemokines, adhesion molecules and cytokines. It also leads to development renal cell damage (Faubel et al., 2007). Inflammatory biomarkers TNF-α and IL-6 cytokines were upregulated in cisplatin treated HEK-293 cells and found to get significantly ($p < 0.05$) reduced by treating with extract and swertiamerin in cisplatin treated cells.

The increase in ROS production leads to oxidative DNA damage which contributes to p53 activation in mesangial (glomerular) cells followed by apoptosis (Ju et al., 2014). Flow cytometric mensuration of ROS level indicates that ROS level got increased in cisplatin treated HEK-293 cells and ameliorates significantly by extract and swertiamerin treatment.

Cell cycle inhibitory drugs like apigenin (Ju et al., 2015), roscovitine and olomoucine (Price et al. 2004) have evinced to protect kidney cells in vitro from apoptosis. Moreover, the cell cycle inhibition also plays an effective measure against cisplatin nephrotoxicity (Megyesi et al., 1998). The extract was also examined for the cell cycle inhibition in nephrotoxic cells. Distribution of cells were observed in various stages of the mitotic phase M, the DNA synthetic phase S, and the pre- and post-DNA synthetic phases, G1 and G2, respectively. Extract and swertiamerin treatment after cisplatin administration enhances the percentage of cells in G1 and S phase this justifies the extract and swertiamerin decelerate the cell proliferation rate and permit the cell to repair itself before entering the cell cycle. Thus, extract and swertiamerin prevent uncontrolled proliferation and allows the cells to repair

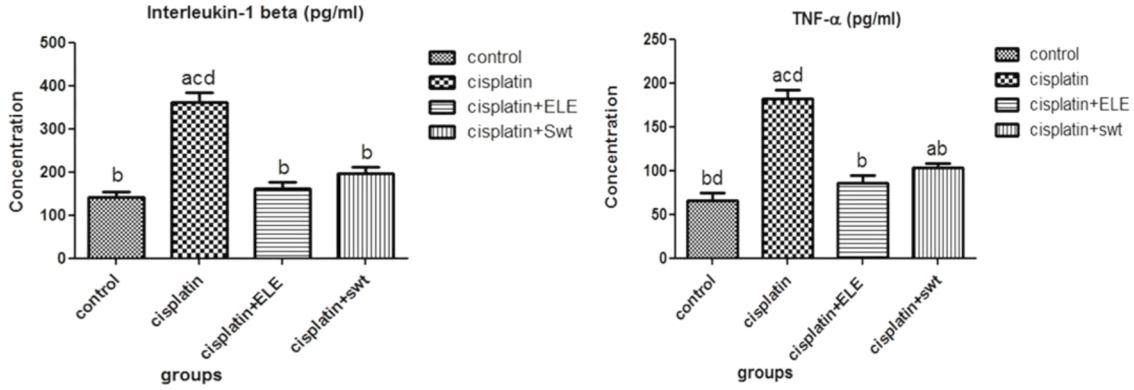

Fig. 6. Graph signifies elevation in proinflammatory cytokines (IL-1β and TNF-α) level in HEK-293 cells after cisplatin administration. ELE and swertiamerin treatment group showed lowering in proinflammatory cytokines level to the normal significantly ($p < 0.05$).
Values expressed as means ± SEM ($n = 2$), a: $p < 0.05$, comparing with control group (vehicle); b: $p < 0.05$, comparing with toxic control group (cisplatin); c: comparing with ELE treatment group 50 μl (2 mg/ml); d: $p < 0.05$, comparing with swertiamerin 20 μl (0.5 mg/ml), (One way analysis of variance (ANOVA) followed by Newman–Keuls Multiple Comparision test).

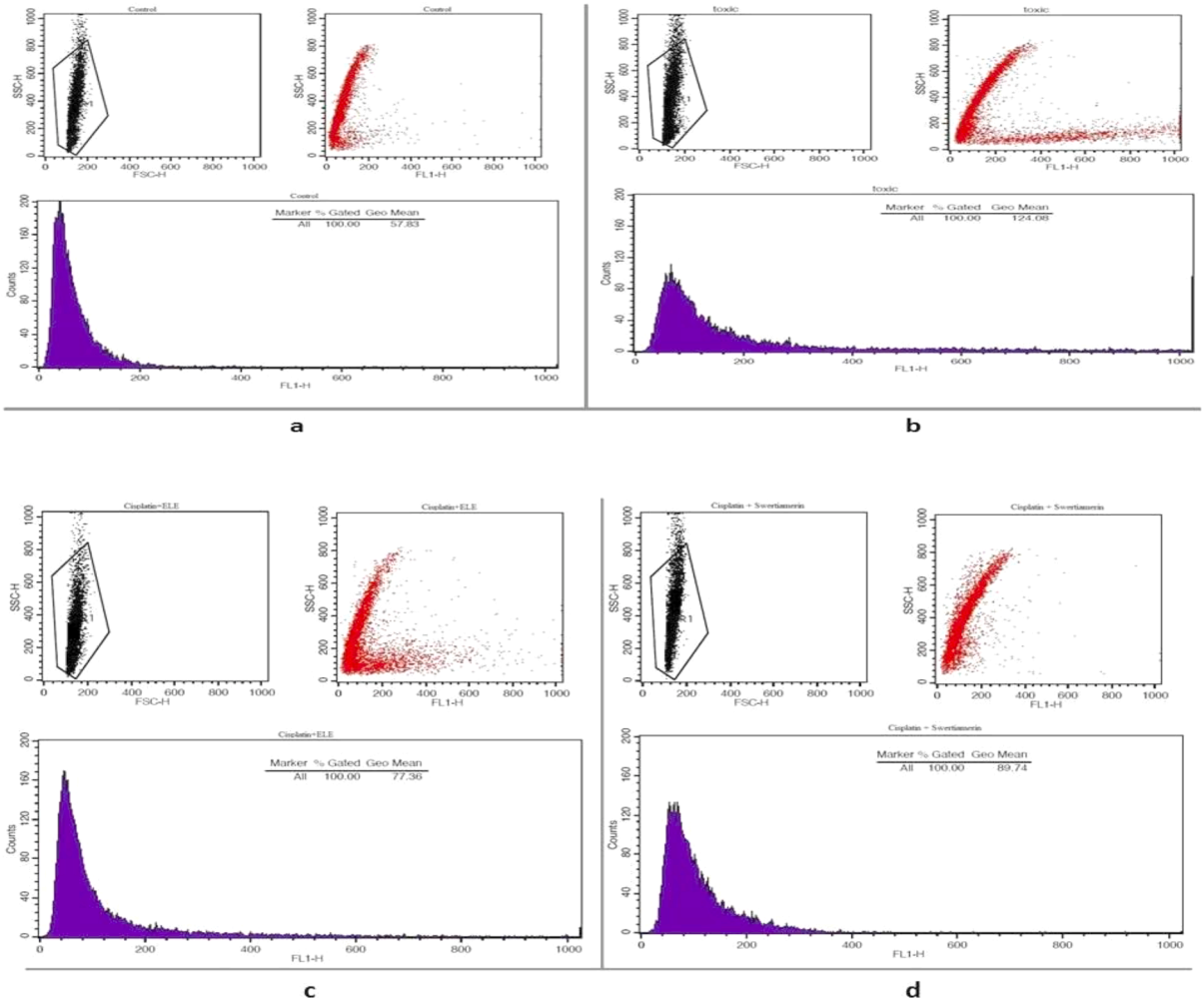

Fig. 7. Flow cytometry analysis of intracellular ROS production by HEK-293 cells, a. untreated cells (Geo mean 57.63) b. HEK-293 cells after exposure to cisplatin alone (Geo mean 124.08) c. ELE treated cells after Cisplatin induced toxicity (Geo mean 77.36) d. Swertiamerin treated cells after Cisplatin induced toxicity (Geo mean 89.74).

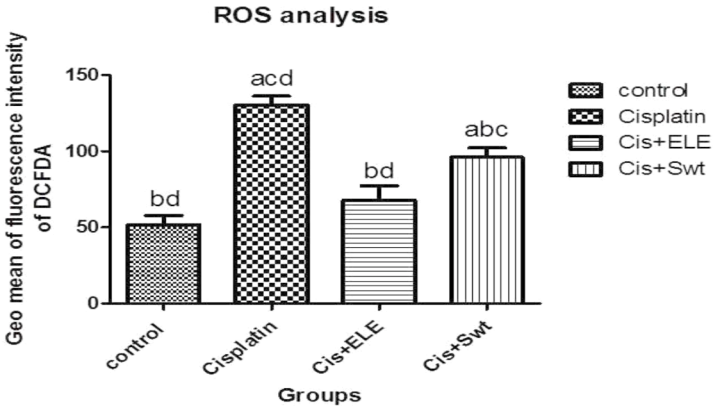

Fig. 8. Graph showing Geometrical mean (GeoMean) values of the fluorescence intensities of respective groups for ROS measurement.
Values expressed as means ± SEM ($n = 2$), a: $p < 0.05$, comparing with control group; b: $p < 0.05$, comparing with toxic control group; c: comparing with treatment group (2 mg/ml); d: $p < 0.05$, comparing with swertiamerin (0.5 mg/ml), (One way analysis of variance (ANOVA) followed by Newman–Keuls Multiple Comparision test).

themselves.

DNA damage was monitored through DNA fragmentation assay by gel electrophoresis in HEK-293 treatment cells. It was observed that the DNA damage was initiated by various pathogenic events after cisplatin treatment. DNA damage was controlled by extract and swertiamerin treatment as no fragments were observed.

The findings established the extract to be effective against cisplatin induced nephrotoxicity owing to presence of many bioactive constituents along with swertiamerin mainly phenols, flavonoids (quercetin), terpenoids (ursolic acid), Vitamin C and Vitamin E which possess the nephroprotective properties (Sharma et al., 2017). Swertiamerin might be the important contributing component that confers the pharmacological action.

Consequently, the present study upholds the endogenous medicinal

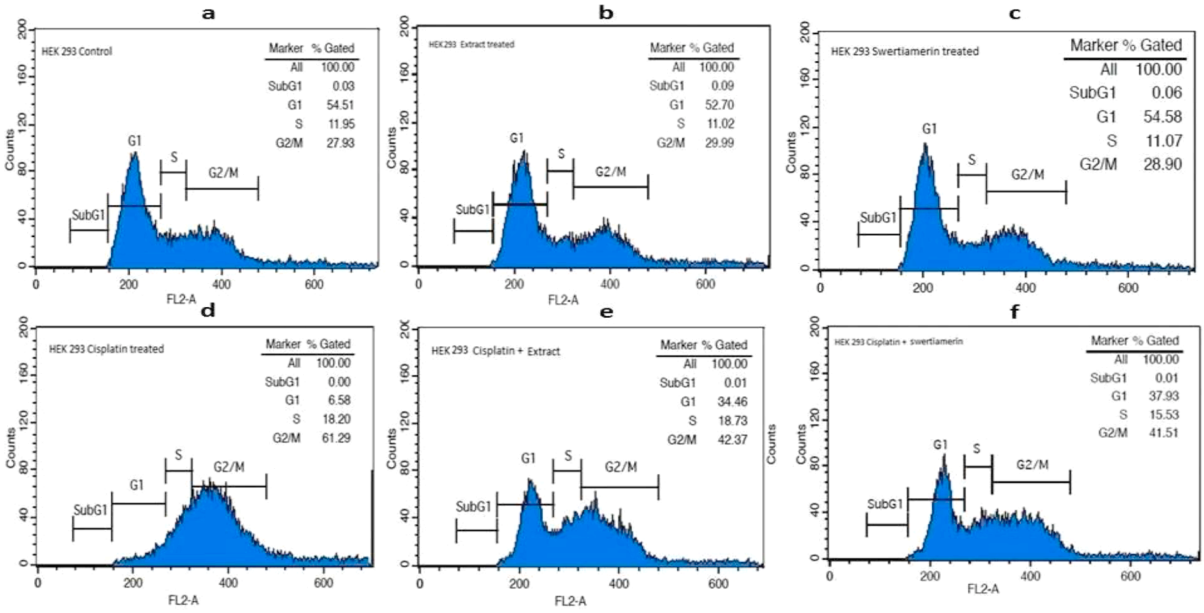

Fig. 9. Flowcytometric analysis showing distribution of HEK-293 cell line in various phases of cell cycle, a: untreated cells, b: ELE treated cells c: swertiamerin treated cells, d: Cisplatin treated cells, e: cisplatin + ELE 50 µl (2 mg/ml) treated, f: cisplatin + Swertiamerin 20 µl (0.5 mg/ml) treated.

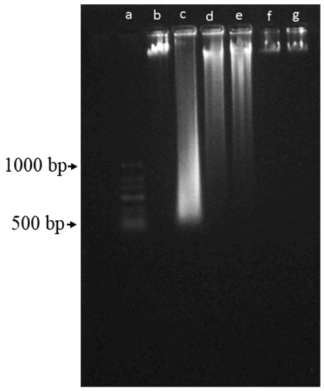

Fig. 10. DNA fragmentation of HEK-293 cells exposed to cisplatin. Each lane reflecting the presence of DNA fragments was viewed on an ethidium bromide-stained gel. Lane a: Marker, Lane b: DNA of untreated cells, Lane c: DNA of Cisplatin treated, Lane d: DNA of cisplatin + ELE 50 µl (2 mg/ml) treated cells, Lane e: DNA of cisplatin + Swertiamerin 20 µl (0.5 mg/ml) treated, Lane f: DNA of ELE 50 µl (2 mg/ml) treated alone, Lane g: DNA of 20 µl Swertiamerin (0.5 mg/ml) treated alone.

uses of *Exacum lawii* in kidney problems by using modern scientific techniques. Furthermore, the present study gives the scientific basis for understanding the mechanism of action of *Exacum lawii* in cisplatin treated HEK-293 cells.

The nephrotoxicity is the common side effect of anticancer drug treatment. So, the present study helps to seek attention for developing herbal drug for the treatment of kidney toxicity. It is also suggested to understand the importance of traditional herbal medicine which needed to be conserve and further research is needed to develop them for clinical use.

5. Conclusion

The current research work scientifically validates the ethnopharmacological use of *Exacum lawii* and proved it as an efficient and promising new therapeutic agent for preventing nephrotoxicity. In the present research, molecular docking study of swertiamerin (marker compound in the extract) and *in-vitro* assays in HEK-293 cell line were designed to determine the mechanism of action for it nephroprotective activity. Moreover, this research also provides information regarding the involvement of iNOS enzyme, reactive oxygen species, pro-inflammatory cytokines in development of cisplatin induced cellular injury. This study can provide valuable insights for developing the herbal drug which prevents the nephrotoxicity as major side effect from anti-cancer drugs. Furthermore, the study is required to develop a potent herbal formulation from the standardized *Exacum lawii* extract and its clinical evaluation.

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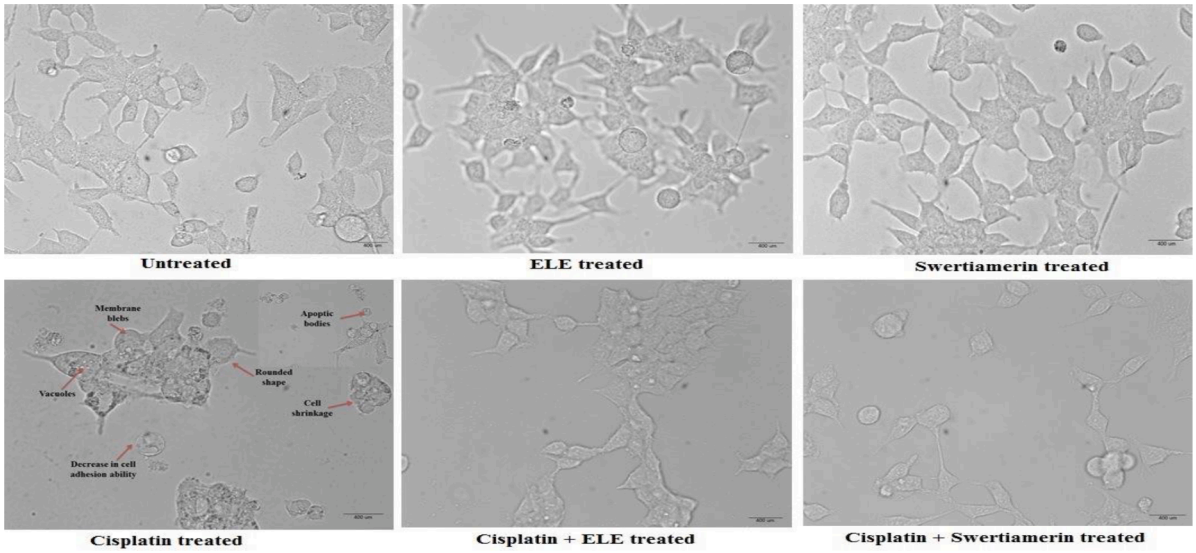

Fig. 11. Morphology of HEK-293 cell line in untreated condition, treatment with ELE alone and swertiamerin alone showed adherent, flattened, and elongated morphology. Cisplatin exposure causes cells less adherent, produce apoptic bodies, formation of blebs, shrinkage of cell membrane. Cells with ELE treatment and swertiamerin treatment respectively after cisplatin administration recover the toxic symptoms and conserve the morphology.

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Nil

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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