PHARMACOGNOSTICAL EVALUATIONS

1. Plant authentication and extraction process

The whole herbs of *Exacum lawii* were collected in the month of August-October from Mahabaleshwar, Maharashtra, India. The herbs were identified and authenticated by Dr. N M Dongarwar, Assistant Professor, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, India. A voucher specimen (Cog/EL/2014-15) of *Exacum lawii* was deposited for future reference in pharmacognosy laboratory of Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi, India.

The freshly collected herbs were thoroughly washed and shade dried for one week at temperature not exceeding 60ºC to prevent the denaturation of thermos-labile phytoconstituents. The dried herbs were coarsely powdered using mechanical grinder, passed through 60 mesh sieve size and stored at room temperature for powder study and physiochemical parameters. The powdered drug (1000 gm) was defatted with petroleum ether (60-80ºC) and exhaustively extracted by cold maceration using 95% ethanol (3 liter v/v) as solvent. The *Exacum lawii* extract (ELE) was filtered and concentrated up to 50 ml using vacuum rotary evaporator (IKA Germany) and stored in vacuum desiccator until use. The obtained ethanolic extract of *Exacum lawii* (ELE) was then fractionated using different solvents mainly toluene, chloroform, ethyl acetate and water. The extract and its fractions were then evaluated for colour, taste and extractive value.

2. Morphological, microscopical and powder evaluation

Morphological, microscopical and powder evaluation were performed according to standard procedure (Khandelwal, 2007; Evans, 2002). The morphological evaluation

involved the size, texture, colour, shape, odour and taste of whole herb. For microscopical study, whole herb was preserved in n-butyl alcohol. The transverse sections were taken with the help of rotary microtome (York Scientific Industries Pvt. Ltd.) and sections were dehydrated with different ratio of absolute alcohol and then stained with phloroglucinol-HCl. Finally, the obtained stained sections were permanently mounted with DPX for histological study (Brain and Turner, 1975).

Sections of whole herb were also analyzed by SEM (scanning electron microscope - quanta 200) at low vacuum. The detector used was secondary electron detector. The pressure was maintained in the range of 5.99 to $6.02e^{-1}$ torr. The sections were placed on a circular aluminum specimen stub, pre-coated with silver glue (for increasing conductivity of electrons) and placed over observation area. It was then observed under the SEM in varying magnifications and micrographs were recorded.

Powdered drug was stained with phloroglucinol-HCl and observed under microscope by placing in microscope slide. For the study of macerated tissue and isolated cells, the whole herb was treated with the concentrated nitric acid and potassium chlorate for overnight. After washing the tissues with distilled water finally mounted in glycerine and covered with the coverslip for observation under microscope. Photographs of different magnifications were taken and with Nikon Trinocular Microscopic unit, Model E-200, Japan. Quantitative microscopy was done by using anatomical section and the epidermal layer of fresh leaves (Johansen, 1940).

3. Random amplified polymorphic DNA (RAPD) fingerprinting profile

3.1. DNA isolation, Quantification and Electrophoresis

The weighed quantity of freshly collected plants of *Exacum lawii* was flashed with liquid nitrogen followed by grinding of frozen plants using mortar and pestle. Grounded plant samples were transferred in eppendorf, to the samples in eppendorf, Proteinase-k (3mg/ml) was added. These samples were incubated at 37°C for one hour in a water bath shaker. Pre-warmed extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 2.5 M NaCl, 3 % CTAB, 1 % Sarkosyl and 1 % 2-mercaptoethanol (v/v)) was added after incubation and again kept in shaker water bath at 65°C. The supernatant (5 ml) were separated and transferred to each tube and allowed to cool at room temperature followed with equal volume chloroform: isoamyl alcohol (24:1) solution. An upper aqueous and a lower organic solvent layer in each tube were observed. Tubes were slowly inverted up-down without shearing of DNA until homogenous emulsion was formed. The tubes were centrifuged at 4ºC for 15 minutes at 9200 rcf using 3K30 laboratory centrifuge. The upper transparent aqueous layer containing nucleic acid was safely transferred to another 50 ml tube using a 5 ml micropipette with broad bore tips. Double volume of pre-chilled ethanol and 3M sodium acetate (pH 5.2) was added to each tube. The tubes were left over night at −20°C for nucleic acid precipitation. The tubes were centrifuged at 20,000 rcf for 15 minutes to pellet the precipitated nucleic acid. The supernatant was removed and pellets obtained were washed in 70 % ethanol. The pellets were dried in hot air blower for complete removal of ethanol. These pellets were suspended in 50 µl of TE- buffer (EDTA 1 mM and Tris-HCl 10 mM pH 8.0). The suspension was incubated with

pancreatic RNase-A (0.2 µg/µl) for 2 hours at 37°C to remove RNA content. The resulting suspensions contained DNA isolated from *Exacum lawii* was stored at -20°C.

The concentration of DNA was checked by measuring A_{260}/A_{280} ratio in a Hitachi UV-Vis spectrophotometer. Isolated DNA was electrophoresed in 0.8 % agarose gel containing 1 µg/ml ethidium bromide using TAE (40 mM Tris-acetate and 1 mM EDTA of pH 8.0) electrophoresis buffer and photographed in the gel documentation system (Bio-Rad, USA).

3.2. RAPD-PCR and electrophoresis

Six different primers were selected to generate reproducible amplification products (Bafeel *et al*., 2012; Sameera *et al*., 2011). Sequences of the primers (Sigma grade) used for RAPD-PCR are given in table 5. PCR was performed in 25 µl reaction mixture containing 40 ng DNA, 2.5 µl of 10X PCR buffer (500 µM dNTPs, 4.5 mM $MgCl_2$, 0.5µM of each primer and 0.6 unit per µl Taq DNA polymerase (Bangalore Genie, India) in an I-cycler PCR thermal cycler (Bio-Rad, USA). The thermal cycler profile was as follows: initial denaturation for 1 minute at 95°C followed by 35 incubation cycles each consisting of 94°C for 30 seconds denaturation, 30 seconds annealing at 48°C then 60 seconds at 68°C and a final 5 min elongation at 68°C. Primers were combined in an equimolar ratio for the multiplex reaction. PCR products were separated by electrophoresis in 1.5 % (w/v) agarose gel using TAE buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8.0) containing 0.5 µg/ml ethidium bromide and photographed. RAPD banding profiles were analyzed by using an Amersham

100-bp ladder on the basis of presence or absence of the bands of a particular size were determined by utilizing fingerprinting II version-3 software (Bio-Rad, USA).

Table 5: Primers used for amplification of matK and rbcL gene segments

Primer name	**Primer set**	**Binding**	**Primer sequence (5´– 3´)**
matK-2.1F	matK-A	Forward	CCTATCCATCTGGAAATCTTAG
matK-5R		Reverse	GTTCTAGCACAAGAAAGTCG
rbcLaF	rbcL-A	Forward	ATGTCACCACAAACAGAGACTAAAGC
rbcLaR		Forward	GTAAAATCAAGTCCACCRCG
rbcL-1F	rbcL-B	Reverse	ATGTCACCACAAACAGAAAC
rbcL-724R		Reverse	TCGCATGTACCTGCAGTAGC

4. Nutritional content analysis and physicochemical Evaluation

Nutritional contents analysis defines the content of vitamins, minerals, fatty acid profile with its composition and calories obtained from fat, was determined using standard methods. The dried powdered plant material was carried for further physicochemical parameters like extractive value, moisture content, swelling index, water soluble ash, total ash, acid insoluble ash, pesticide residue, foaming index, bulk density, pH, volatile oil content (by distillation method), foreign matter, heavy metal content, crude fiber content and volatile oil determination as per WHO guidelines (Horwitz, 2003; WHO 2002). Each experiment was performed in triplicate, mean values with standard error of the mean (SEM) were calculated.

4.1. Foreign matter

The coarsely powdered air dried herb (100 gm) was evenly spread on white paper sheet and was carefully observed with the help of magnifying glass. Foreign material such as sand, clay and other particles were removed from the plant material and was further weighed. The foreign matter in plant material was calculated as percentage weight/weight (% w/w).

4.2. Total ash

Accurately weighed (2 gm) of air dried coarsely powdered plant material was incineration in the tarred silica dish at the temperature not exceeding 450°C until it became in form of white ash and free from carbon. The total ash was allowed to cool, weighed and signify as percentage (% w/w) with reference to air dried drug.

4.3. Acid-insoluble ash

Total ash in the crucible obtained as a result of incinerating powdered plant material was weighed and 25 ml of hydrochloric acid (70 gm/l) was added followed by gentle boiling for 5 min. The solution was then filtered using a weighed ash-less filter paper and was later washed with warm water to make it neutral. The filter paper containing the insoluble matter was dried, ignited and further cooled in a desiccator. The content was then weighed and percentage of acid insoluble ash was measured with reference to the air dried plant material (% w/w).

4.4. Water-soluble ash

The silica crucible containing the total ash was added with 25 ml of water and was boiled for 5 minutes. The insoluble matter was allowed to filter using weighed ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature

not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the total ash taken and the percentage (% w/w) of water soluble ash was measured.

4.5. Loss on Drying

Previously weighed (1gm) dried plant material was taken in a shallow shaped glass-stoppered weighing bottle and was evenly distributed by gentle sidewise shaking to a depth not exceeding 10 mm and dried in temperature not exceeding 105°C and for specified time to constant weight. After achieving constant weight the bottle was cooled by placing in desiccators. It was then weighed and the percentage loss on drying was calculated (% w/w).

4.6. Foaming Index

The accurately weighed (1 gm) air-dried powdered drug was transferred into a conical flask (500 ml) containing 100 ml of boiling water. The mixture was allowed to boil for 30 minutes, cooled and filtered into 100 ml volumetric flask and the volume was made up to the mark with distilled water. The filtrated decoction was then poured into 10 stoppered test-tubes (height 16 cm × diameter 16 mm width) in successive portions of 1 ml to 10 ml and in each tube, the volume was adjusted with water up to 10 ml. The stoppered tubes were closed and were shaken in a lengthwise direction for 15 sec with two shakes per seconds and were then allowed to stand for 15 min. The height of the foam was calculated and results were estimated as follows:

- If the height of the foam in each tube is less than 1 cm, the foaming index is taken to be less than 100.

- If the height of any tube from 1 to 10 cm, then the volume of the decoction in this tube is used to determine the index and is marked as ‘a’ in the formulae given below.
- If the above mentioned tube is the first or second tube in a series, then prepare an intermediate dilution in a similar manner to obtain a more precise result.
- If the height of the foam is more than 1 cm in every tube, the foaming index is over 1000. In this case, experiment must be repeated by using a new series of dilutions of the decoction in order to obtain a result.

The foaming index was calculated using the following formula:

Foaming Index = 1000/a

Where, ‘a’ is the volume in ml of the decoction which used for preparation of the dilutions in the test tube.

4.7. Swelling Index

Swelling index is defined as “the volume (in ml) taken up by the swelling of 1 gm of plant powder under specified condition”. Dried plant material of was weighed (1 gm) accurately and was taken in a 25 ml glass-stoppered measuring cylinder (125 mm length and 16 mm internal diameter). 25 ml of distilled water was added and the mixture was thoroughly shaken after every 10 min interval for 1 hour. It was allowed to stand for 3 hour, at room temperature. The volume occupied by the plant material was measured in ml. The mean value of the individual experiments, with respect to 1 gm of plant material was calculated.

4.8. Determination of crude fiber content (Dutch method)

Crude fiber content is the weight of tissues, residue which can be obtained after giving treatment with dilute acid and alkali to the plant material. It was determined by treating 2 gm of powdered plant material with 50 ml of 10% nitric acid and heated to boiling with constant stirring. The whole material was strained through a fine muslin cloth using Buckner funnel and obtained residue was washed with warmed water and it was further treated with 50 ml 2.5% w/v sodium hydroxide solution and heated to boiling. The whole content was again strained, dried and weighed, finally, percentage (% w/w) of crude fibers was measured (Khandelwal, 2007).

4.9. Fluorescence powder drug analysis

Fluorescence analysis of powdered drug plays an important role in identification and distinguishing one powdered drugs from similar another drug. The fluorescence analysis was carried out according to the standard method (Kokoski *et al*, 1958; Chase and Pratt, 1949). The fluorescence pattern of the powdered drug solution when mixed with different solvents was visualized under day light as well as under short (254 nm) and long ultra violet light (365 nm).

5. Pesticide residue and Heavy metal estimation

The pesticide residue content was determined as per the methods described by the WHO guidelines. The 50 gm of coarsely powdered plant was mixed with solvent system of 350 ml of acetonitrile: water (65:35) at high speed for 5 min and then filtered. The obtained filtrate (250 ml) was transferred to a separating funnel containing 100 ml of petroleum ether, 10 ml of sodium chloride (40%) and 600 ml of

distilled water, followed by constant shaking up to 35-45 seconds. Aqueous layer was discarded from the organic layer and the later was washed twice with 100 ml of water then 15 gm of anhydrous sodium sulfate was added with vigorous shaking and volume was reduced up to 5-10 ml. The concentrated volume was then subjected directly to column packed with florisil R 60/100 PR grade, activated at 650° C. Three different eluate were obtained using three different ratios of petroleum ether and diethyl ether mixture as mobile phase like first eluate contained (94:6) while second eluate of petroleum ether and diethyl ether (85:15) and third eluate with 50% mixture of petroleum ether and diethyl ether. Elution was done at 5 ml per minute. All the eluates obtained were transferred to a silica sample holder were concentrated and subjected to combustion. Combustion was done in a one liter conical flask followed with oxygen, fused with a platinum wire (1mm) attached with a platinum gauze (1.5 × 2 cm) for purpose of holding the sample. The sample holder was made up of halide free filter paper (5 cm long and 3 cm wide). The gases produced gets absorbed in a suitable solution in the combustion flask (water for chloride and H_2SO_4 in case of phosphate pesticides). In case of chloride pesticides, 15 ml of the solution obtained after combustion was mixed with 1 ml of ferric ammonium sulfate (0.25 mol/l) and 3 ml of mercuric thiocyanate followed by agitation and absorbance was measured at 460 nm. For determining phosphate pesticides, 0.4 ml of ammonium molybdate (40 gm/l), 2.2 ml of sulfuric acid (300 gm/l) and 0.4 ml of amino-naphtholsulfonic acid was mixed with 7 ml of the sample obtained after combustion followed by spinning and heating at 100°C for 12 minutes finally the absorbance was measured at 820 nm.

Heavy metals content analysis involved estimation of four important heavy metals *viz.* Arsenic (As), Lead (Pb), Mercury (Hg) and Cadmium (Cd). Two gram of powdered

drug was treated with 10 ml concentrated nitric acid followed by heating in a hot plate for 15 min at 95°C. This process was repeated twice for another 30 minutes until the red fumes of nitric acid disappear. The solution was cooled to room temperature and 2ml of deionized water and 3ml hydrogen peroxide (30% v/v) was added to start the peroxide reaction. After the reaction was completed, add 5ml of concentrated hydrochloric acid followed with addition of 10 ml of deionized water and heat the sample for further 15 minutes. The sample was cooled, filtered and volume was made up to 50 ml. The estimation of heavy metal content was analyzed by using atomic absorption spectroscopy (Shimadzu–AA6300). Data obtained was expressed in terms of parts per million (ppm), (1ppm=1mg/kg) (Gomez *et al*., 2007).

6. *In vitro* antioxidant activity

6.1. Free radical scavenging activity

The free radical scavenging activity of ELE and its fractions were evaluated by employing the DPPH (1, 1-diphenyl-2-picryl-hydrazil) assay method (Blois, 1958). According to protocol, 100 µM/ml solution of DPPH was prepared in methanol and its 5 ml of solution was added to 1ml of ELE and its various fractions in different concentrations. After thirty minutes of incubation, the absorbance was taken at 517 nm. The free radical scavenging activity was calculated by using the equation (1):

$$\text{DPPH scavenging effect} = [(1-A_1/A_0)] \times 100 \quad \ldots\ldots\ldots\ldots\ldots (1)$$

Where A_0 is the absorbance of the blank and A_1 is the absorbance of the test sample. The percentage inhibition was plotted against respective concentrations used and IC_{50}

was calculated using ascorbic acid as control. The experiment was performed in triplicate.

6.2. Nitric oxide scavenging assay

Nitric oxide scavenging assay was performed by using sodium nitroprusside assay (Sreejayan and Rao, 1997). Nitric oxide generated from Sodium nitroprusside reacts with oxygen to produce nitrite ions. This can be determined by the use of the Griess Illosvoy reaction. Sodium nitroprusside (2 ml,10 mM) in 0.5 ml phosphate buffer saline (pH 7.4) was added to 0.5 ml of extract and its fraction at various concentrations and the mixture was incubated for 150 min at 25°C. The 0.5 ml of incubated mixture was taken out and added to 1.0 ml of 33% sulfanilic acid reagent in 20% glacial acetic acid and incubated for 5 min at room temperature. At last, 1.0 ml naphthylethylenediamine dihydrochloride (0.1% w/v) was added to mixture and kept at room temperature for 30 minutes. The absorbance was measured at 546 nm. The nitric oxide radical scavenging activity was calculated according to equation (1). The IC_{50} was calculated by using rutin as the positive control. All readings were performed in triplicates.

6.3. Scavenging of hydrogen peroxide

Hydrogen peroxide radical scavenging activity by using hydrogen peroxide was done according to standard procedure (Jayaprakasha *et al*., 2004). A solution of hydrogen peroxide (20 mM) was prepared using phosphate buffered saline (PBS, pH 7.4). Hydrogen peroxide solution (2 ml) in PBS was added to 1 ml of different concentrations of ELE/fractions and standard in methanol. After 10 minutes, the

absorbance was measured at 230 nm. All readings were performed in triplicates and the percentage inhibition was calculated using above equation (1).

6.4. Scavenging of hydroxyl radical by deoxyribose method

Scavenging activity of the ELE and its fractions by hydroxyl radical was measured by degradation of Deoxyribose method (Halliwell *et al*., 1987). Ferric chloride (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH, 7.4, 20 mM) was added to the 0.2 ml of various concentrations of the extract and its fractions. The reaction mixture was then incubated for 30 min at 37^{o}C. After incubation, ice-cold trichloroacetic acid (0.2 ml, 15%, w/v) and thiobarbituric acid (0.2 ml, 1%, w/v) in 0.25N HCl were mixed. The reaction mixture was heated in boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm against a blank containing phosphate buffer. The percentage inhibition was calculated using equation (1).

PHYTOCHEMICAL EVALUATIONS

1. Preliminary Phytochemical screening

Preliminary phytochemical screening of ELE and its various fractions was performed to evaluate the presence of various classes of phytochemicals like alkaloids, glycosides, flavonoids, tannins, steroids/triterpenoids, phenolics, bitter constituents, saponins, mucilages, protein, carbohydrates and amino acids as per standard procedures (Evans, 2002; Harbone, 1984).

1.1. Test for alkaloids

Mayer's test

Test solution when treated with freshly prepared Mayer's reagent results in formation of cream colored precipitate, it ensures the presence of alkaloids.

Dragendroff's test

Estimated volume of the test solution when treated with Dragendroff's reagent leads to formation of orange to reddish colored precipitate in sample confirming the presence of alkaloids.

Wagner's test

When Wagner's reagent is added to test solutions, it leads to formation of reddish brown colored precipitate ensuring the presence of alkaloids in test solution.

1.2. Test for phytosterols/steroids

Libermann Burchard test

Acetic anhydride (3 ml) was added to the test solution. To this mixture, two drops of

conc. sulphuric acid was added slowly along the wall of the test tube. Appearance of bluish green colour confirms the presence of phytosterols/steroids.

1.3. Test for anthraquinones glycosides

Borntrager's test

In this test, the extract was first shaken gently with equal volume of chloroform and then the chloroform layer was separated. To this layer dilute ammonium solution was added and if ammonia layer acquires pink colour, it confirms the presence of anthraquinone glycosides.

Modified Borntrager's test

To the extract, 5% ferric chloride solution and dilute hydrochloric acid was added, followed by heating on boiling water bath for 5 minutes to bring about oxidative hydrolysis and then shaken gently with addition of benzene. Benzene layer was further separated and dilute ammonia solution was added to the solution. Formation of pink colour indicates the presence of anthraquinone glycosides.

1.4. Test for cardiac glycoside

Legal's test

To the test solution, few drops of sodium nitroprusside and 1 ml of pyridine were administered and mixture was made alkaline with NaOH solution. Appearance of pink is an indicative of presence of cardiac glycosides.

1.5. Test for coumarins glycoside

The 10 % of dilute NaOH was added to the test sample, and the mixture was observed under UV light (366 nm). Appearance of blue colour fluorescence from reaction mixture confirms the presence of coumarin glycoside in test sample.

1.6. Test for cytogenetic glycosides

Sodium picrate test

Sodium picrate paper is prepared by soaking filter paper first with 10% picric acid and then with 10% sodium carbonate. Sodium picrate paper when comes in contact with the extract containing cyanogenetic glycosides turns in to brick red colour.

1.7. Test for Phenolic compound

The extract dissolved in methanol is the test sample, the few drops of freshly prepared solution of 5% ferric chloride was added to sample. Formation of blue colour indicates the presence of phenol.

1.8. Test for tannins

To the test solution (extract dissolved in methanol), 0.5 ml of 10% lead acetate was added. Appearance of white precipitate indicates the presence of tannins in the sample.

1.9. Test for flavonoids

To the test solution prepared by dissolving extract in methanol, required amount of

magnesium turnings and a few drops of concentrated hydrochloric acid were added. Formation of pink colour indicates the presence of flavonoids.

1.10. Test for saponins

To the extract, 1 ml of distilled water was added and shaken vigorously in the test tube. Formation of persistent foam/froth for 10 min or more indicates the presence of saponins.

1.11. Test for carbohydrates

Molisch's test

In this test, the test sample was prepared to react with 0.5 ml of α-naphthol solution. The mixture was vortexed and two drops of concentrated H_2SO_4 was added carefully from the side of the test tube. Formation of purple ring at the junction of two liquid phases showed the presence of carbohydrate.

1.12. Test for proteins

Biuret test

Test solutions were added with 40% sodium hydroxide and dilute copper sulphate solution. Formation of blue colour indicates the presence of proteins in sample.

Xanthoproteic test

The test solutions were treated with concentrated nitric acid and boiled for few seconds. Formation of yellow colour precipitate indicates presence of proteins in sample.

1.13. Test for amino acids

To the test solution, 2 ml of ninhydrin solution was added drop wise and the solution was heated and if violet colour observed it indicates the presence of amino acids in sample.

2. Quantitative estimation

2.1. Total phenolic content

The total phenolic content of extract was done according to Folin-Ciocalteu assay. In volumetric flask of 10 ml, 1 ml of extract was mixed with 8 ml of water. To this 0.5 ml of folin-Ciocalteu reagent was added after 15 minutes followed by addition of 1.5 ml of 20 % sodium carbonate solution. After incubation period of 2 hours the absorbance of coloured reaction product was measured at 765 nm at ambient temperature. Calibration curve was prepared by using different concentration of gallic acid as standard. All calculations were performed in triplicates. Results were expressed as mg of gallic acid equivalent per gram of dried extract (Grubesic *et al*., 2005).

2.2 . Total flavonoid content

For determining total flavonoid content aluminium chloride method was used. 100 µl of extract (10 mg/ml) was added in 100 µl of 20% aluminium trichloride in methanol followed with addition of drops of acetic acid and the volume was made up to 5 ml with methanol and incubated for 40 minutes. After incubation period, absorbance was measured at 415 nm. Different dilutions of rutin as standard were taken for making

calibration curve. All calculations were carried out in triplicates. The amount of flavonoid in extract was calculated by using formula.

$X= (A \times m_o) / (A_o \times m)$

Where 'X' is flavonoid content (mg/g) of plant extract in RE. 'A' and 'A_o' is the absorbance of extract and standard rutin respectively. 'm' is weight of crude extract in mg and 'm_o' is weight of rutin in mg. The results were expressed in rutin equivalent (RE) (Kumaran and Karunakaran, 2006).

2.3. Total flavonol content

The total flavonol content was also determined by Aluminium chloride method with some modifications using rutin as standard. 1 ml of crude extract (10 mg/ml) was mixed with aluminium trichloride (20 mg/ml) followed with addition of 3 ml of sodium acetate (50 mg/ml). After incubation of 2.5 hours, the absorbance was measured at 440 nm. All the measurements were taken in triplicates (Kumaran and Karunakaran, 2006).

2.4. Total alkaloid content

The 5 gram of plant powder was taken in 250 ml beaker and 200 ml of 20% acetic acid was added and covered for 4 hours. This mixture was filtered and the volume was reduced to one quarter in vacuum rotary evaporator. To this solution, concentrated ammonium hydroxide was added drop-wise until the precipitate was formed completely. The whole precipitate was allowed to settle down at bottom. The precipitate was strained, collected and weighed (Biju *et al*., 2014).

3. Thin Layer chromatography

Phytochemical analysis using thin layer chromatography (TLC) was performed to evaluate the presence of various classes of phytoconstituents in ELE and its fractions. TLC analysis was done according to standard procedure by using precoated aluminium silica gel plates 60F–254 as stationary phase. Solvents having varying polarity in different ratio as described in standard procedure were used as mobile phase for developing the chromatogram. Developed plates were visualized initially under day light and also under UV-254 or UV-365 nm, under which chromophoric groups of phytoconstituent shows quenching of fluorescence. Different spraying reagents like dragendorff reagent for alkaloids, vanillin sulphuric acid and fast red salt B for the presence of bitter principle drugs, fast blue salt reagent followed by 10% KOH for polyphenolics classes, Liebermann-Burchard reagent for saponins, ninhydrin reagent for amino acids and steroidal components to confirm the presence of various phytoconstituents present in extract or its fractions. The presence of chemotaxonomic marker swertiamerin in the ethanolic extract of *Exacum lawii* was ensured by Co-TLC along with the standard swertiamarin (Natural remedies Pvt Ltd) using solvent system ethyl acetate: methanol: water (7.7: 1.5: 0.5) (Wagner and Bladt, 1996).

4. HPLC standardization with chemotaxonomic marker Swertiamerin

HPLC analysis was carried out by using Waters 1500-series pump (Milford, MA, USA) attached to Waters 2998 photodiode array detector and data were analysed using waters Breeze software (Waters, USA). The mobile phase and sample were filtered through a 0.45 μm membrane filter by solvent filtration apparatus (Millipore, USA). Analysis of the samples was carried out in Column Lichro CART® 250-4 C18

column and methanol-water (1:1) to get better separation, peak shape and resolution at UV 238 nm and thus used for further analysis.

The chromatographic conditions for swertiamerin: flow gradient started with flow rate of 0.5 ml/min. At 4.9 minute, the flow rate was increased to 0.8 ml/min then increased to 1.0 ml/min at 5.1 min. The flow rate was kept constant up to 5.4 min and then finally decreased to 0.5 ml/min, restoring the initial conditions at 8 min. Detector's wavelength was 238 nm and the injection volume was 10 µl. The best base line separation (peak purity >95%) for swertiamarin was achieved at 238 nm also reported earlier. Swertiamarin was found to show maximum absorption at λ max 238 nm in three-dimensional ultraviolet absorption spectra using photo diode array (PDA) detector (Rana *et al*., 2012)

5. GCMS analysis of *Exacum lawii* extract

GC-MS analysis of the ethanolic extract of *Exacum lawii* was performed from Arbro pharmaceutical Ltd. Delhi, by Shimadzu GCMS-QP-2010 plus system. RTx-5 Sil MS column (internal diameter 30 m × 0.25 mm, 0.25 m film thickness) was used for the analysis. Helium was used as the carrier gas. The injector temperature was maintained at 270°C, Oven temperature programmed from 80°C to 210°C at 4°C /min from 210°C to 300°C in the interval time of 2 minutes and at 15°C /min at interval of 5 minutes, and the final temperature was made for 20 minutes. Pressure 85.4 kPa, the volume of injected sample was 0.2 litre, column flow 1.21 ml/min, total flow 76.8 ml/min, linear velocity 40.5 cm/sec, purge flow 3.0 ml/min, split ratio: 60.0; The MS operating conditions were: m/z 40-600; ion source temperature 230°C; and interface line temperature 28°C. *Identification of the compounds*: Identification of compounds

was based on the interpretation of the mass spectral fragmentation followed by comparing their mass spectra with data from standard data, NIST05 (National Institute of Standards and Technology, US) and WILEY 8 libraries by comparing with the MS literature data (Jennings and Shibamoto, 1980; Adams, 2007).

6. Isolation of Swertiamerin

The dried plant powder (500 gm) was defatted with petroleum ether followed by extraction in ethanol. The obtained ethanolic extract was filtered and concentrated. The obtained concentrated extract was treated with cold diethyl ether and the precipitate was obtained. The precipitate was triturated with silica gel (60-120 mesh, E. Merck, Germany) and loaded on a column. Initially eluted with petroleum ether (60-80 °C) and then with mixture of petroleum ether and ethyl acetate (1-20%) followed by ethyl acetate and then with ethyl acetate containing increasing amounts of methanol (0-12%). Different elutes were monitored by thin-layer chromatography (TLC) for swertiamarin using the solvent system of ethyl acetate: methanol: water (7.7: 1.5: 0.5). Elutes which showed single spot in TLC were collected and pooled together. The presence of swertiamarin was confirmed by co-chromatography with standard swertiamarin (Natural remedies Pvt Ltd). Recrystallization and purification were done by dissolving the residue in methanol (Vishwakarma *et al*., 2004).

6.1. Identification of swertiamerin

Swertiamerin was identified by determining its melting point using open capillary tubes on a Stuart melting point apparatus (SMP10). UV absorption spectrum (Shimadzu (1700) Double beam) and Infrared absorption spectrum using FT-IR

spectrophotometer (Shimadzu, model 8400, Tokyo) of isolated swertiamerin were recorded. Data after anlysis was compared with literature.

6.2. Structure elucidation of swertiamarin

The structure of swertiamarin was elucidated by ^{1}H NMR (300 MHz and 500 MHz) and ^{13}C NMR (75 MHz and 125 MHz), 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 thermo fisher LCMS with ion trap ESI-MASS detector. The data obtained was analysed and compared with literature (Rai and Thakar, 1966; Naggar and Beal 1980; Anwar *et al*., 1996).

7. Molecular Docking Study

7.1. Protein structure 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 minimized. Virtual screening and molecular docking study of swertiamerin as selective inhibitor of inducible nitric oxide synthase enzyme was performed. The docking study was performed using AutoDock 4.2 package software to investigate the affinity of swertiamarin to the binding pocket of iNOS.

7.2. Ligand preparation

Swertiamarin was downloaded as mol2 file from zinc database and the energies of compound were minimized. The compound was converted into the PDB format using open Babel 2.3.1 software.

7.3. Receptor Grid generation

During the docking, the grid dimensions were 50X48X54 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 2,500,000 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 favorable free energy of binding were selected as the resultant complex structures. All calculations were carried out on PC based machines running Windows 7, 32 bit as operating system. The resultant structure files were analysed using Discovery Studio Visualizer 3.1 obtained from (www.accelerys.com).

PHARMACOLOGICAL EVALUATION

1. Nephroprotective activity of *Exacum lawii* extract (ELE) and swertiamerin against cisplatin induced nephrotoxicity in experimental rats

1.1. Animals

The certified pathogen free healthy Charles Foster albino male and female rats (150–250 gm) were procured from the Central Animal House (Reg. No. 542/02/ab/CPCSEA), Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Maximum three rats were kept in one polypropylene cage and maintained under standard circumstances (12 hour light and dark cycles at an ambient temperature of 25℃ and 45–55% relative humidity). Rats were permitted for free access to water and standard feed. The animals were allowed to acclimatize to the laboratory conditions for seven days prior to the commencement of experiments. All experimental protocols were approved by Central Animal Ethical Committee of Banaras Hindu University (No. Dean/2015/CAEC/1132) and were conducted according to the accepted standard guidelines of National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication no. 85–23, revised 1985).

1.2. Toxicity profile of ELE

1.2.1. Acute oral toxicity study

As per OECD guidelines 420, the study was conducted by using a limit dose of 2000 mg/kg. The administration of ELE showed no mortality or any toxic symptoms at dose of 2000 mg/kg throughout the observation period of 14 days. There were no significant changes found in body weight change, heart rate, breathing, behavior,

sensory nervous system response, cutaneous effect, gastrointestinal effect and locomotor behavior during and after the period of observation.

1.2.2. Repeated dose 28-days oral toxicity study

Repeated dose 28-days oral toxicity study in experimental rats was performed in accordance with OECD test guideline 407. For each group nulliparous and non-pregnant 5 female and 5 healthy male rats were taken. The first group was taken as control group, given with normal saline. Second, third and fourth group were administered with ELE at dose of 1000 mg/kg, p.o., 2000 mg/kg, p.o. and 4000 mg/kg, p.o. to overnight fasted rats. The observations were conducted individually for each rat, any neurological and behavioural changes such as convulsions, tremors, diarrhoea, sleep, feeding, salivation and lacrimation behaviour considered as sign of acute toxicity for 48 hours. Morbidity and mortality were observed daily for 28 days. Preclinical signs, delayed occurrences and reversibility, of signs of toxicity, were distinguished, for at least 14 days post treatment. body weight and organ weight of rats in respective groups were compared with rats in control group. Further blood was collected by retro-orbital bleeding under anaesthesia to estimate biochemical parameters by using span diagnostic ltd and haematological parameters by mythic 18 haematology analyser. Histopathological examination was performed for vital organs of the body.

1.3. Dose standardisation of cisplatin

To standardize the dose of cisplatin for inducing nephrotoxicity in albino rats, three groups each having six rats in each group were taken. Cisplatin (0.9% w/v suspension

of sodium chloride) (Cytoplatin-10, Cipla) at a dose of 4 mg/kg, 6 mg/kg, and 8 mg/kg was administered by intraperitoneal injection to rats in respective group. After 72 hours of cisplatin treatment, blood was collected through retro orbital venous plexus under light anaesthesia. Serum urea and creatinine level were measured by using standard kits (Erba diagnostics Mannheim, Germany).

1.4. Drug treatment protocol

To evaluate the nephroprotective activity of ELE and swertiamerin against cisplatin-induced nephrotoxicity, rats were divided equally into six groups containing six rats each. The ELE was suspended in 0.5% CMC and given by per-oral administration (p.o.) to rats. Cisplatin (0.9% NaCl suspension) was administered by intraperitoneal injection (i.p.). The doses of ELE were selected as $1/10^{th}$ of the safe dose obtained from toxicity study. The pilot study was performed for selecting the dose of swertiamerin, the lowest effective dose (20 mg/kg, p.o.) was selected (Gui *et al*., 2013). Different doses of ELE (100 mg/kg, p.o., 200 mg/kg, p.o., 400 mg/kg, p.o.) and swertiamerin (20 mg/kg, p.o.) were given for total 7 days to respective groups and cisplatin (6 mg/kg, i.p) was administered on the 4^{th} day of the treatment. Finally on 7th day, rats were sacrificed to collect blood and harvest kidneys for measuring various parameters (renal function test, liver function test, antioxidant enzymes and proinflammatory cytokines level), estimation of reactive oxygen species in single viable cells of treated kidney, DNA fragmentation assay to evaluate the degree of DNA damage in kidney cell treated with cisplatin and histological studies (Figure 7).

Group I (normal control): vehicle (aqueous solution of 0.5% CMC) for 7 consecutive days and 0.9% NaCl on the 4th day.

Group II (Toxic control): vehicle (aqueous solution of 0.5% CMC) for 7 consecutive days and cisplatin (6 mg/kg, i.p.) on the 4th day.

Group III: ELE (100 mg/kg, p.o.) for 7 consecutive days and cisplatin (6 mg/kg, i.p.) on the 4th day.

Group IV: ELE (200 mg/kg, p.o.) for 7 consecutive days and cisplatin (6 mg/kg, i.p.) on the 4th day.

Group V: ELE (400 mg/kg, p.o.) for 7 consecutive days and cisplatin (6 mg/kg, i.p.) on the 4th day.

Group VI: Swertiamerin (20mg/kg, p.o.) for 7 consecutive days and cisplatin (6 mg/kg, i.p.) on the 4th day.

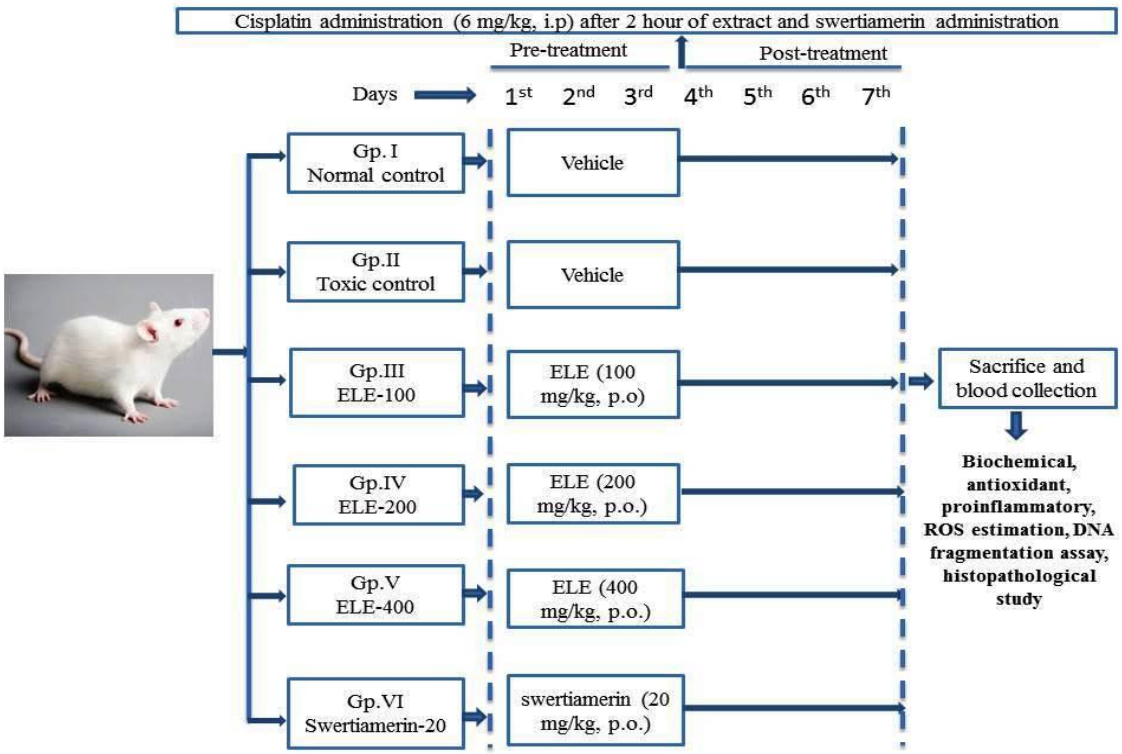

Figure 7: Drug treatment protocol in rats for evaluation of nephroprotective activity

1.5. Determination of biochemical parameters in serum

Enzyme assay kits were purchased from span diagnostic limited. Serum urea (Fawcett and Scott, 1960) and serum creatinine (Larsen, 1972) had been measured using BUN method and modified rate Jaffe's kinetic method by autoanalyser. Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT) (Schumann *et al*., 2002), Total protein (Doumas *et al*., 1971), Total bilirubin (TB), Direct bilirubin (DB) (Garber, 1981) and alkaline phosphate (ALP) (Kind and King, 1972) had been also analyzed according to standard protocols using the automated analyzer.

1.6. Determination of antioxidant parameters in renal tissue homogenate

Minced kidney tissues were homogenized in 0.1 M potassium phosphate buffer (pH-7) with the protease inhibitor. Obtained homogenates (10% w/v) were centrifuged (10,000 x g) at 4ºC for 20 minutes and used for detection of antioxidant parameters.

1.6.1. Estimation of Lipid peroxidation (LPO)

Lipid peroxidation in homogenized kidney tissues after treatment was estimated by the protocol described by Ohkawa *et al*., 1979.

Principle

Malondialdehyde (MDA), a secondary product of LPO reacts with thiobarbituric acid (TBA) in order to form a pink chromogen (Thiobarbituric acid-2 malondialdehyde adduct), which is measured by spectrometrical method.

Reagents

a) 8.1% of Sodium dodecyl sulphate (SDS) (Sigma) - 810 mg SDS dissolved in 10 ml distilled water.

b) 20% Acetic acid (Merck) - 20 ml of Glacial acetic acid added to 80 ml of distilled water (freshly prepared). The pH of the solution was adjusted to 3.5 by adding NaOH.

c) 0.8% Thio-barbituric acid (Sigma) - 800 mg TBA dissolved in 100 ml of distilled water.

d) Butanol and Pyridine mixture (15:1) (Merck) - 15 ml of n-butanol was mixed with 1 ml of pyridine.

e) 1, 1, 3, 3-tetramethoxypropane (Sigma) was used as standard for LPO estimation.

Procedure

LPO level was measured in terms of malondialdehyde (MDA). To 0.2 ml of 100 mg/ml kidney tissue homogenate 0.1 ml of 8.1 % SDS, 0.75 ml of 20 % acetic acid solution (pH 3.5) and 0.8 % aqueous solution of TBA (0.75 ml) was added in stoppered tubes. The volume was made up to 2 ml of distilled water, and then heated in a water bath at 95°C for 60 minutes. After cooling with tap water, 0.5 ml of distilled water and 2.5 ml of mixture of pyridine and n-butanol (1:15) were added and shaken vigorously. After centrifugation at 3000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured against blank containing 0.2 ml of distilled water in place of sample. 1, 1, 3, 3- tetramethoxypropane was used as external standard and the level of LPO was expressed as nmol MDA/gm of wet tissue.

1.6.2. Estimation of Catalase (CAT)

Catalase activity in the homogenized kidney tissues was done by the method described by Sinha, 1972.

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2), resulting in the formation of per-chromic acid as an unstable intermediate, the chromic acetate, later produced is measured spectrophotometrically.

Reagents

a) Disodium hydrogen phosphate

b) Phosphate buffer (0.01M; pH-7.0)

c) potassium dihydrogen orthophosphate

d) Potassium dichromate ($K_2Cr_2O_7.7H_2O$-5%)

e) Hydrogen peroxide (H_2O_2-0.2M)

f) Glacial acetic acid

Procedure

The kidney tissue homogenate was mixed with phosphate buffer (1.0 ml) followed by addition of H_2O_2 for initiation of the reaction. The reaction was arrested immediately by addition of 2.0 ml dichromate- acetic acid reagent at 0, 30 and 60 seconds intervals. The blank reagent was prepared by addition of 1.6 ml of buffer and 2.0 ml of dichromate acetic acid reagent taken in separate tubes. The test solutions and blank tubes were heated for 10 min in boiling water bath to develop green colour. The tubes

were cooled to room temperature and their absorbance was measured at 570 nm using spectrophotometer against the blank. Results obtained were expressed as µmol H_2O_2 consumed/min/mg of protein.

1.6.3. Estimation of Reduced Glutathione (GSH)

To estimate GSH level in homogenized kidney tissues, Sedlak and Lindsay *et al*., 1968 method was followed.

Principle

DTNB is a disulphide compound which is reduced by sulfhydryl group of reduced glutathione (GSH) and produces yellow colour. The produced yellow colour was measured by spectrophotometer at 412 nm.

Reagents

a) 50 % Trichloro acetic acid (TCA) - 10 gm TCA added to 20 ml distilled water.

b) 0.4M Tris buffer (Sigma) - Prepared by dissolving 4.84 gm of Tris in small amount of distilled water and by adding 10 ml of 0.2M EDTA and make up to 100 ml with distilled water. Adjust the pH to 8.9 with 1N HCl.

c) 0.2M Ethylene diamine tetra acetic acid (Merck) - 7.4 gm of sodium salt of EDTA is dissolved in distilled water to make it a volume of 100 ml.

d) 0.02M EDTA - Take 10 ml of 0.2M EDTA and make up to 100 ml with distilled water.

e) 0.1M Di-thio bis nitro benzoic acid (Ellman's reagent) (Hi-Media) - Dissolve 9.9 mg of DTNB ($C_{14}H_8N_2O_8S_2$) in 25 ml of absolute methanol.

f) Reduced glutathione ($C_{10}H_{17}N_3O_6S$) was used as the standard.

Procedure

Tissue homogenate (100 mg/ml), 1ml was mixed in 15 ml test tube with 0.8 ml of distilled water and 0.2 mL of 50 % TCA. The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at 3000 rpm for 10 min. 0.6 ml of supernatant was mixed with 0.8 ml of 0.4M Tris buffer (pH 8.9) and 20 µl of 0.1M DTNB in absolute methanol, and the sample was shaken. The absorbance was taken in 5 minutes of the addition of 40 µL DTNB at 412 nm against a reagent blank with no homogenate. The data were expressed as nmol/gm of wet tissue and were calculated from the standard curve prepared by using standard glutathione.

1.6.4. Estimation of Superoxide dismutase (SOD)

Superoxide dismutase in granulation tissue was estimated by method described by Kakkar *et al*., 1984.

Principle

The SOD assay of is based on principle of the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction is extracted into butanol and measured at 560nm.

Reagents

a) 0.052M Sodium pyrophosphate (Sigma) - 1.16 gm of SPP added in 50 ml of distilled water and the pH was adjusted to 8.3

b) 186 µM Phenazine methosulphate (Sigma) - 18 µl of 100mM of PMS added to 10 ml of distilled water. PMS (100mM) prepared by adding 306 mg in 10 ml of distilled water

c) 300 µM Nitroblue Tetrazolium (Sigma) - 2.754 mg of NBT dissolved in 10 mL of distilled water

d) Nicotinamide adenine dinucleotide (Sigma) (780 µM) - 11.1 mg of NADH dissolved in 20 ml of distilled water

e) Glacial Acetic acid

f) n-Butanol

Procedure

The inhibition of reduction of nitro-blue tetrazolium (NBT) to blue colour formazan in presence of phenazine metha-sulphate (PMS) and Nicotinamide adenine dinucleotide (NADH) was measured at 560 nm using blank as n-butanol. To 0.2 ml of tissue homogenate was added 0.6 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3), 150 µl of 300 µM NBT, 50 µl of 186 µM of PMS and 0.4 ml of distilled water to make up the volume up to 1.5 ml including with 0.1 ml of 780 µM NADH. Reaction was started by the addition of NADH. After incubation at 30°C for 60 sec, the reaction was stopped by the adding 0.5 ml of glacial acetic acid. The reaction mixture was shaken and stirred vigorously with 2 ml of n-Butanol. The mixture was allowed to stand for 10 min, centrifuged for 10 min at 3000 rpm and butanol layer was taken out. Colour intensity of the chromogen in the butanol was measured at 560 nm in spectrophotometer against n-butanol as blank, system devoid of enzyme served as control. One unit of enzyme activity is defined as enzyme concentration required for inhibiting the optical density at 560 nm of chromogen by 50% in one min under the standard conditions and the results have been expressed as units (IU) of SOD activity/gm wet tissue.

1.7. Determination of proinflammatory in renal tissue homogenate

Cisplatin accumulation in kidney tissues led to the generation of proinflammatory cytokines due to toxicity induced and had been investigated by measuring levels of TNF α, IL-6 and IL-1β on homogenized renal tissues using ELISA kit (Komabiotech, Korea). Standard and detection antibodies provided were reconstituted in sterile water. Serial dilutions of samples and standard were prepared. Selected wells in microplate were washed with washing solution. 100 ml of samples and standard were added to wells followed by addition of diluted detection antibody after incubation. Colour was developed with the addition of colour development enzyme and colour development solution. Finally, absorbance of developed colour was measured by using microplate reader (BioTek instruments Inc. USA).

1.8. Flow-Cytometric Measurement of Reactive oxygen species (ROS)

Renal single cell preparation

Rats were anaesthetized with diethyl ether and sacrificed. Kidneys were isolated washed with 1X PBS and minced into small pieces with fine scissor. The minced tissues were transferred in 20 ml of 0.1% Type IV collagenase in 1X PBS solution and placed in a CO_2 incubator at 37°C for 2 hours. The cell suspension was passed through cell strainer (40 μm mesh size). The cells were centrifuged at 3000 rpm for 10 minutes, 3 times. The supernatant was discarded and the pellet was suspended in FAC sheath to perform flow cytometric analysis.

Flow-cytometric measurement

FACS Calibur flow cytometer (BD Bioscience) in conjunction Cell-Quest Pro software was used for flow cytometry. 20µM of 2′, 7′-Dichlorofluorescin diacetate (Sigma) was added to the renal single cell suspension and incubated in dark for 15 minutes at 37 degree Celsius. Fluorescence intensity was acquired at FL1 channel with BD FACS calibur (BD Biosciences). 10,000 events were taken and Cell Quest Pro software (Becton Dickinson, Franklin Lakes, New Jersey, USA) was used for analysis.

1.9. Qualitative DNA fragmentation assay

Rat kidneys were washed with 1X PBS, minced tissues were homogenised with 1X PBS buffer. Obtained homogenate were centrifuged 5 times at 3000 rpm for 10 minutes at room temperature, and the supernatant was discarded to remove cell debris and RBCs. The cells were lysed in 50 µl of lysis buffer containing 50 mmol/litre of Tris–HCl (pH 8.0) and 0.5% SDS and incubated for 30 minutes at 37°C. The cell pellet was stirred with a wide-bore pipette tip to ensure uniform mixing of cells, followed by the addition of 2µl of 10 mg/ml DNase free RNase (Thermo scientific^{TM}) and incubated for 2 hour at 37°C. Samples were further appended with 5 µl of Proteinase K (Thermo scientific^{TM}) solution and incubated at 50°C for 90 minutes. The precipitated DNA was dissolved in a 20 µl of Tris-EDTA buffer (1X) and quantified spectrophotometrically as described previously. An equal concentration of DNA was resolved on 1% agarose gel stained with ethidium bromide. The gel was viewed under UV light, followed with documentation using the Alpha Innotech system (San Leandro, California, USA)

1.10. Histopathological study

Isolated kidneys were washed with isotonic saline were fixed in 10% neutral buffered formalin for 48 hours and embedded in paraffin wax. Sections (5–6 µm thickness) had been made from paraffin blocks by microtome and stained with Periodic acid-Schiff reagents (PAS) and subjected to microscopic and imaging system (Original magnification 10X Nikon, Japan) (Tan *et al*., 2008)

2. Screening of *Exacum lawii* extract and swertiamerin for the protection of HEK-293 cell line against cisplatin induced nephrotoxicity

2.1. Cell viability 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) (1µg/ml to 7µg/ml), ELE (2 mg/ml to 14 mg/ml) and swertiamerin (0.5mg/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 hour at 37° C. Viable cells allow to convert the yellow-colored 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.

2.2. 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 fetal bovine serum and 1% penicillin- streptomycin antibiotic combination (Invitrogen Life Technologies, Rockville, USA) at 37°C in a humidified atmosphere with 5% CO2. Trypsin was obtained from HiMedia Laboratories (Mumbai, India). For each experiment (Western Blot Analysis, Pro-inflammatory cytokine, ROS estimation, DNA fragmentation assay, Cell cycle analysis and Morphological analysis) equal number of HEK- 293 cells (1.0x105) was seeded in a 6-well culture plate and labelled. After 24 hours of seeding the cells. HEK-293 cells in one of the 6-well was taken as control (untreated), one of the wells was treated with 3µl of cisplatin (1µg/ml) (toxic control), cells in one well was treated with 50 µl of ELE (2 mg/ml) and after 2 hours, 3µl of cisplatin (1µg/ml) was added and cells in another well was treated with 20 µl of swertiamerin (0.5 mg/ml) and after 2 hours 3µl of cisplatin (1µg/ml) was added. The HEK-293 cells from each treatment group were harvested by trypsinization for further experiments after 48 hours of drug treatment.

2.3. Expression of iNOS by Western Blot Analysis in HEK-293 cells

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 minutes, 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 4ºC for 10 minutes at 12000 rpm and stored at -80 ºC. Equal amount 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-β-actin (sc-47778) antibody was added for overnight at 4˚ C. The membrane was washed thrice with TBST. Anti-iNOS (sc-7271) specific antibodies were added and the membrane was washed with after 3 hours. Finally, immunodetection was performed with dye BCIP-NBT (Sigma, USA) (Mahmood and Yang, 2012).

2.4. Estimation of Pro-inflammatory cytokine

Proinflammatory cytokines levels in protein sample isolated from treated HEK-293 cell line using RIPA buffer had been investigated by measuring levels of TNF-α and IL-6 using standard sandwich Enzyme-Linked Immunosorbent Assay kit (ELISA, Komabiotech, Korea) according to the manufacturer's instruction.

2.5. Estimation of ROS level by Flow-Cytometry

HEK-293 cells were harvested 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 20µM of 2′, 7′-Dichlorofluorescein diacetate (DCFDA, Sigma) was then added and incubated in dark for 15 minutes at 37 ºC. Fluorescence intensity was acquired at FL1 channel with BD FACS calibur (BD Biosciences). Measurements were collected on 10,000 cells per sample and Cell Quest Pro software (Becton Dickinson, Franklin Lakes, New Jersey, USA) was used for analysis.

2.6. Qualitative DNA fragmentation assay

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

2.7. 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 5min 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 minutes at 37°C, followed by staining with 10 µl Propidium iodide (Sigma Aldrich, Bengaluru) (1 mg/ml) for 30 minutes. 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).

2.8. Morphological analysis

When the density of HEK-293 cells in a six well culture plate attains 1×10^5 cells per well for 24 hours under standard culture conditions, the cells were further incubated for 48 hours 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 analyzed using CellSens software (Olympus).

3. Antibacterial and antifungal activity of volatile oil and extract of *Exacum lawii* for the antimicrobial activity against ocular infection

3.1. Test microorganisms

Microorganisms were used to determine the antimicrobial potential of the volatile oil and the plant extracts of *Exacum lawii*. Pathogenic bacterial strains causing ocular infection were: *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Pseudomonas pneumonia*, *Haemophilus influenza*, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis*. Pathogenic fungal strains causing ocular infection were *Candida albicans*, *Candida tropicalis*, *Aspergillus keratitis*, *Fusarium dimerum*. Some other pathogenic bacteria were also used in the investigation *Shigella flexneri*, *Klebsiella pneumonia*, *Serratia marcescens*, *MRSA*, *Morganella morganii*, *Chlamydia trachomatis*, *Plesiomonas shigelloides*, *Shigella boydii*, *Shigella dysentery*, *Shigella sonnei*, *Enterococcus faecalis*, *Vibrio parahaemolyticus*, *Salmonella enterica*, *Proteus*

mirabilis, Salmonella typhi. All cultures were obtained from American Type Culture Collection (ATCC) and preserved at the Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India. The fresh bacterial broth cultures were prepared before the screening procedure.

3.2. Antimicrobial Susceptibility Test

The disk diffusion method for antimicrobial susceptibility test was initially performed to determine the antibacterial activities of the *Exacum lawii* against pathogenic bacteria causing ocular infection. Antibacterial and antifungal activities were screened by disc diffusion method (Kajaria *et al*., 2012; Bhandari *et al*., 2000; Aladag *et al*., 2009). The stock solution was prepared by dissolving volatile oil and 1 gm of plant extract in 100 ml of nuclease free water. The stock solution was serially diluted to prepare various dilutions. The 5 µl from each dilution was dispensed on a sterile disc of Whatman's filter paper no.1 of 6 mm diameter for susceptibility testing. Muller Hinton agar (MHA) plates were prepared by pouring 15 ml of molten media into sterile petriplates. The freshly grown colonies were inoculated in normal sterile saline solution to achieve desired concentration (10^7 cfu/ml). The bacteria were streaked in a radial pattern on the agar plates (Remel™, Thermo Fisher Scientific, USA) (Meyer and Afolayan, 1995). The plates were allowed to dry. The prepared solutions of volatile oil and extract were put on 6 mm sterile disc of Whatman filter paper no. 1. The disc was then placed on the surface of the plate containing medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37 °C for 24 hours for bacteria and 48 hour at 25 °C for fungal agents. At the end of incubation, zone of inhibition were examined. Experiment was done in triplicate.

3.3. Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC)

Standard (Himalaya Optha-care Eye Drop) and test sample of plant extracts and volatile oil were prepared using serial dilution method according to the National Committee for Clinical Laboratory Standards, 2000 (NCCLS, 2000). The test was carried out in 96-well microtiter plates. Equal volumes of each dilution of standard, test sample and nutrient broth were mixed in wells of a microtiter plate. Specifically, 0.1 ml of standardized inoculum (2×10^7 cfu/ml) was added to each well of the microtiter plate. The plates were incubated aerobically at 37^oC for 18-24 hours for bacteria and 48 hours at 25^oC for fungal growth. The lowest concentration (highest dilution) of the standard and test sample that produced no visible bacterial growth (no turbidity) when compared with the control was regarded as the MIC. Further, the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by sub-culturing the test dilution from wells of microtitre plate on to a fresh drug-free solid medium and incubated further for culture (Sharma *et al*., 2016). The highest dilution that yielded no bacterial or fungal colony was taken as the MBC and MFC, respectively.

3.4. 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 being statistically significant. Graph Pad Prism 5.0 software (Graph Pad software, Inc., La Jolla, CA) had been used for all statistical analysis.