PHARMACOGNOSTICAL EVALUATION

1. Plant authentication and extraction process

The plant was identified and authenticated by Dr. N M Dongarwar, Assistant Professor, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, India. The Authentication certificate is attached in appendix 1. Voucher specimen (Cog/EL/2014-15) of *Exacum lawii* was deposited in pharmacognosy laboratory of Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi, India. Extractive value determines the nature and amount of constituents soluble in given solvent. The results are shown in table 6.

Table 6: Extractive value and organoleptic properties of various *Exacum lawii* extracts

Extract	colour	Odour	Taste	Extractive value (%w/w)
Petroleum ether extract	Dark brown	characteristic	bitter	8.48% w/w
Toluene extract	Greenish brown	characteristic	bitter	9.30%w/w
Chloroform extract	greenish	characteristic	bitter	4.56% w/w
Ethyl acetate extract	brownish	characteristic	bitter	1.98% w/w
Ethanol extract	Greenish black	characteristic	bitter	10.70% w/w
Water extract	Dark brown	characteristic	bitter	12.65% w/w

2. Morphological Evaluation

Exacum lawii are annually grown small erect herb (5-15 cm height) and sometimes branched (Figure 8). Leaves are opposite, simple and entire. About 6 mm long, sessile, ovate, acute or sub-obtuse and 3-5 ribbed. Stem is quadrangular (with 4 wings); slender usually simple and branched near the top. Peduncles are 2 to 5 cm

long, usually one flowered. The inflorescence is cymose. Flowers are bisexual and hypogynous. Calyx consists of 4-5 sepals and 4 mm long, ovate and fused at base. Corolla is purplish blue 6-8 mm long, 4-5 lobes and overlapping to the right in bud. Androecium: Stamens are 4-5, filament 1.5 mm long and filiform. Anthers are yellow 1 mm long and oblong. Gynoecium: Ovary is two celled 0.2-0.4 cm long and style was long. Ovules many in each cell, stigma is small and subcapitate. Capsule brown, globose 2 valved capsule 3 mm diameter. Seeds are minute and subcuboid.

Figure 8: Freshly collected *Exacum lawii* whole plant

2.1. Microscopical Evaluation

Midrib in cross-section showed plano-convex shape with a depression on middle of dorsal side. The epidermis is composed of single layer, thin walled and rectangular shaped cells on upper and lower surface of leaf. Trichomes are absent on either side of epidermis. The vascular bundles projects towards dorsal side. The vessels are

arranged in small radial rows. There are circular to isodiametric parenchymatous cells around the vascular bundles. Isobilateral leaf, mesophyll is composed of spongy parenchymatous cells without intercellular space. The mesophyll of lamina consists of 4-6 layered spongy parenchyma cells. Stomata commonly ranunculaceous usually confined to the lower epidermis of leaf (Figure 9A).

Stem is provided with 4 ribs or wing (quadrangular). The epidermis of stem is papillose and unilayer with compressed isodiametric cells followed with 3-6 layered cortex composed of parenchymatic cells. Cortex is followed by single layer endodermis. Underneath, the endodermis there is single layered sclerenchymatic pericyclic cells. Secondary phloem is near pericycle and protoxylem towards pith. Xylem vessels are arranged radially in row towards the pith. Intraxylary phloem is present in the form of continuous ring beneath the vessels. Pith is comprised of rounded or polygonal thin walled parenchymatous cells with no intercellular spaces (Figure 9B).

The root system is small and thin. Root hairs are absent. Outer surface consists of single thin layer of epidermal cells. Cortex is multilayered without intercellular spaces. Endodermis was well distinguished and consisted of longitudinally elongated cells. Secondary xylem is well developed. These cells also showed radial symmetry (Figure 9C-9D).

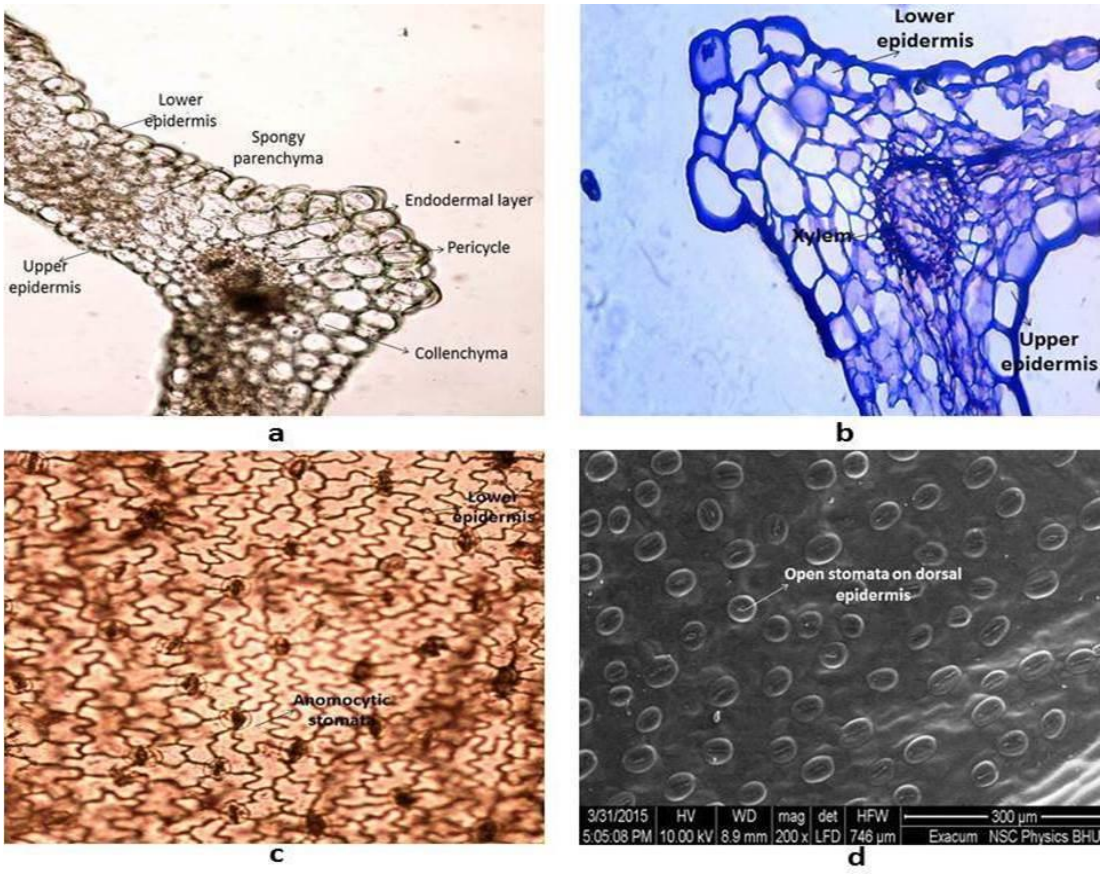


Figure 9A: a and b) Transverse section of midrib, c and d) Stomata on lower epidermis.

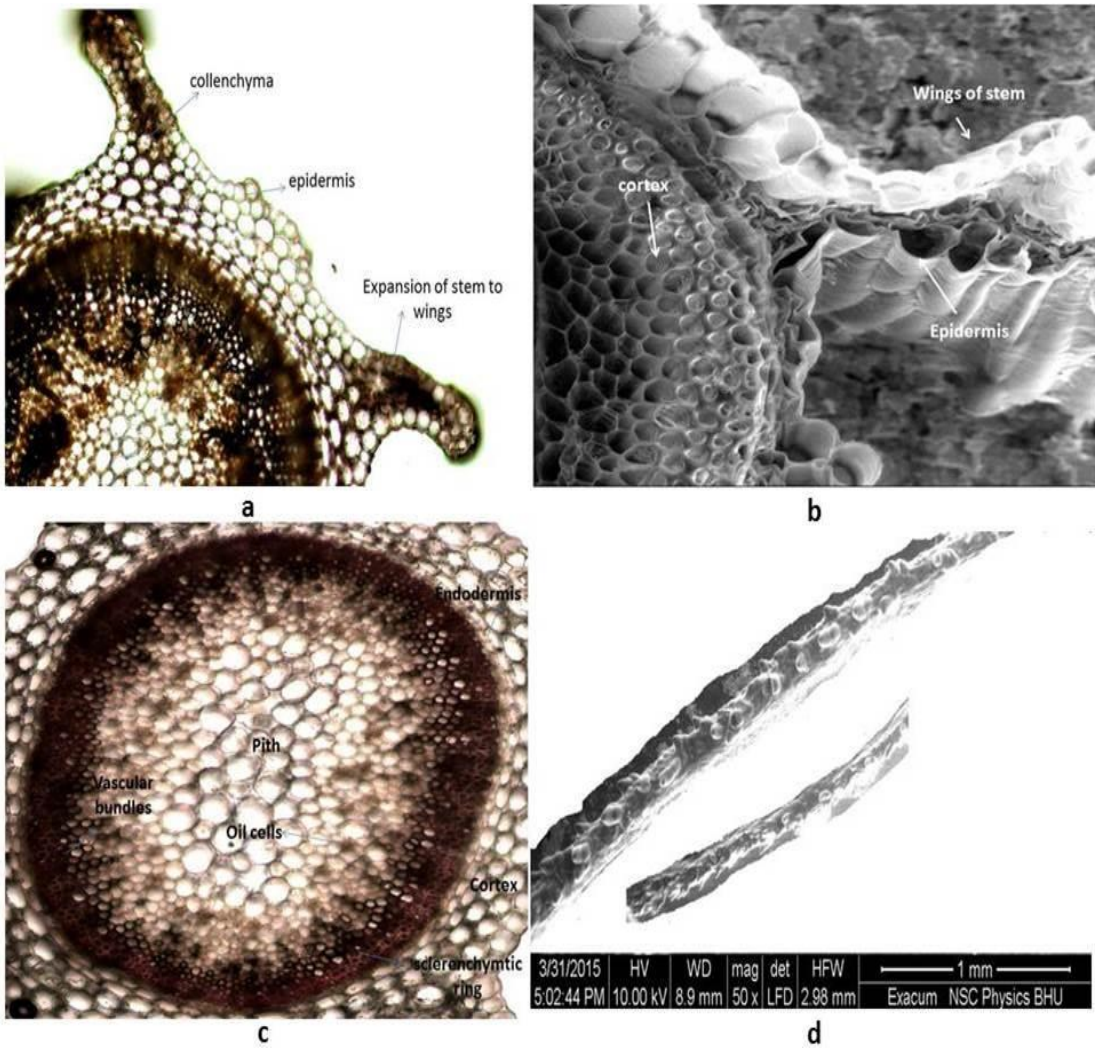


Figure 9B: a) Transverse section of stem showing wings, b) SEM micrograph of stem, c) Transverse section of stem showed centre cylinder, d) SEM micrograph of papillose stem.

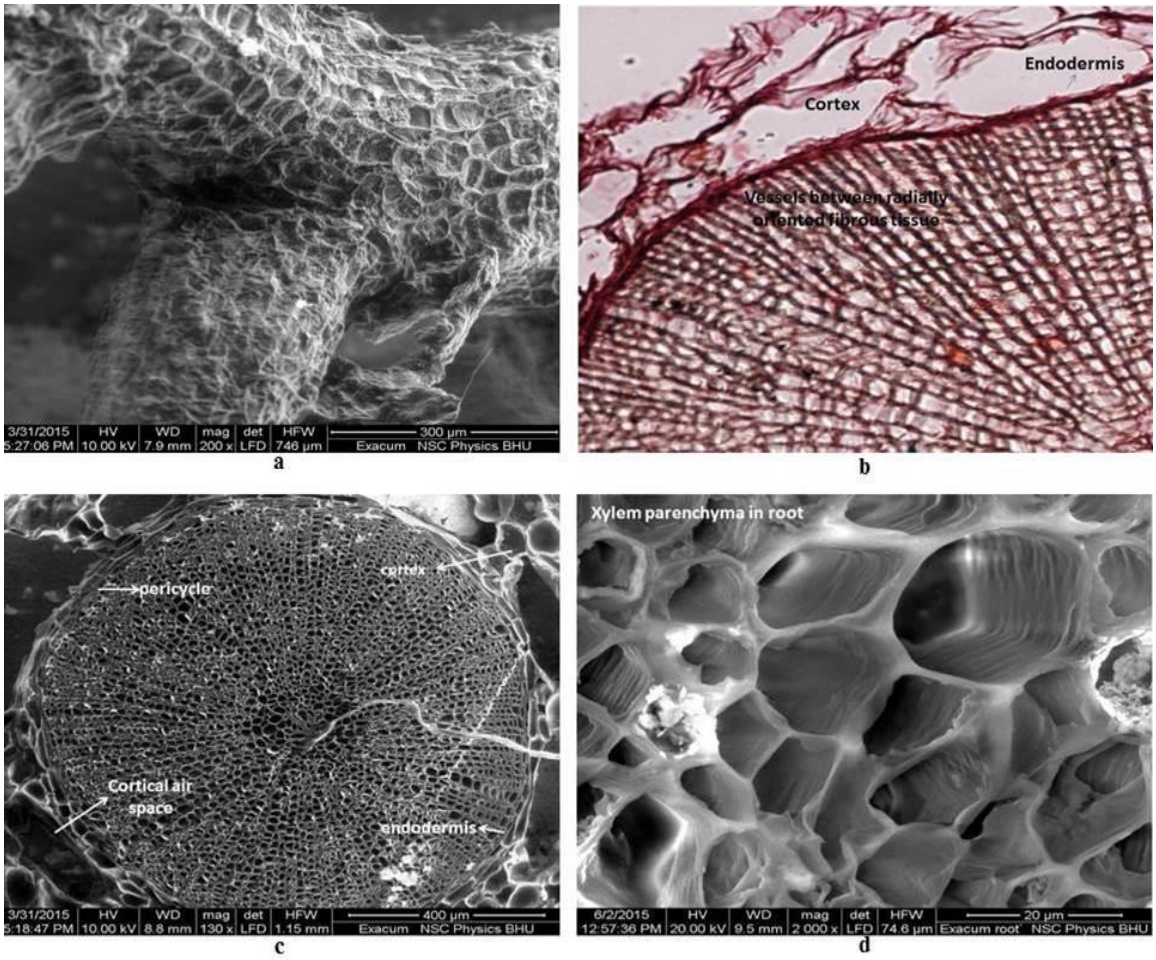


Figure 9C: a) SEM micrograph of root outer surface, b) Transverse section of root cortex, c and d) SEM micrograph of root with radial arrangement of xylem vessels

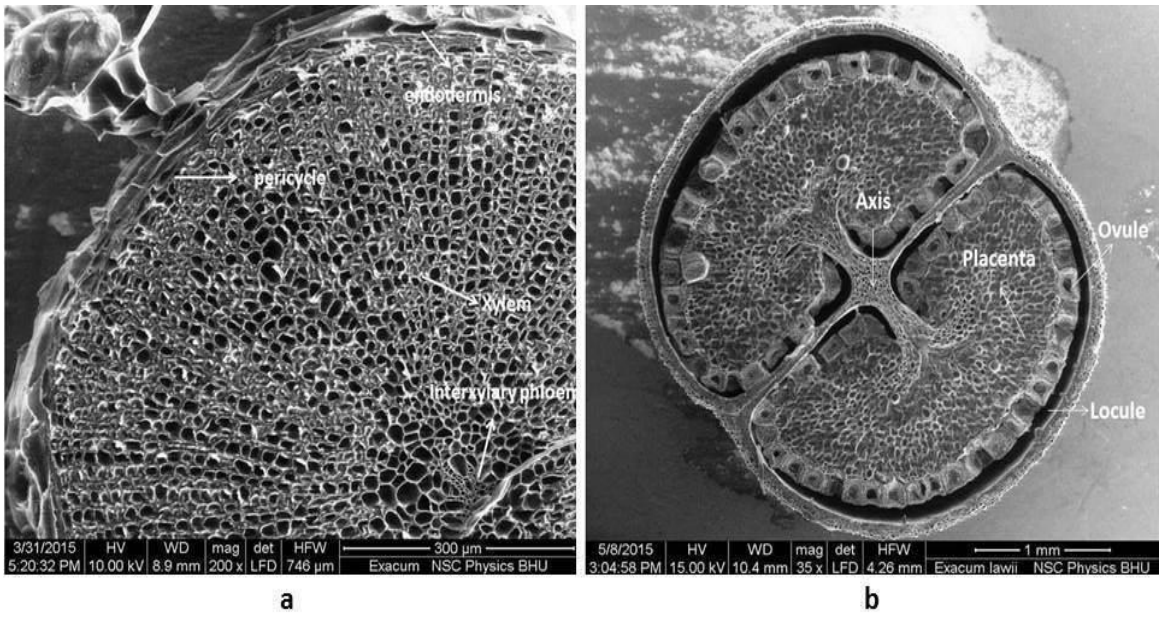


Figure 9D: a) SEM micrograph of root b) SEM micrograph of ovary

2.2. Powder microscopy

Dried powder of whole plant of *Exacum lawii* was investigated under microscope. Astrosclereides, cell wall was drawn out into lobes to form a more or less stellate body and calcium oxalate crystals which play defensive role against herbivores were observed. Presence of parenchymatic cells, brick-shaped cork cells and epidermal cells, spiral xylem vessels, phloem fibres, narrow fibres and starch grains were confirmed (Figure 10).

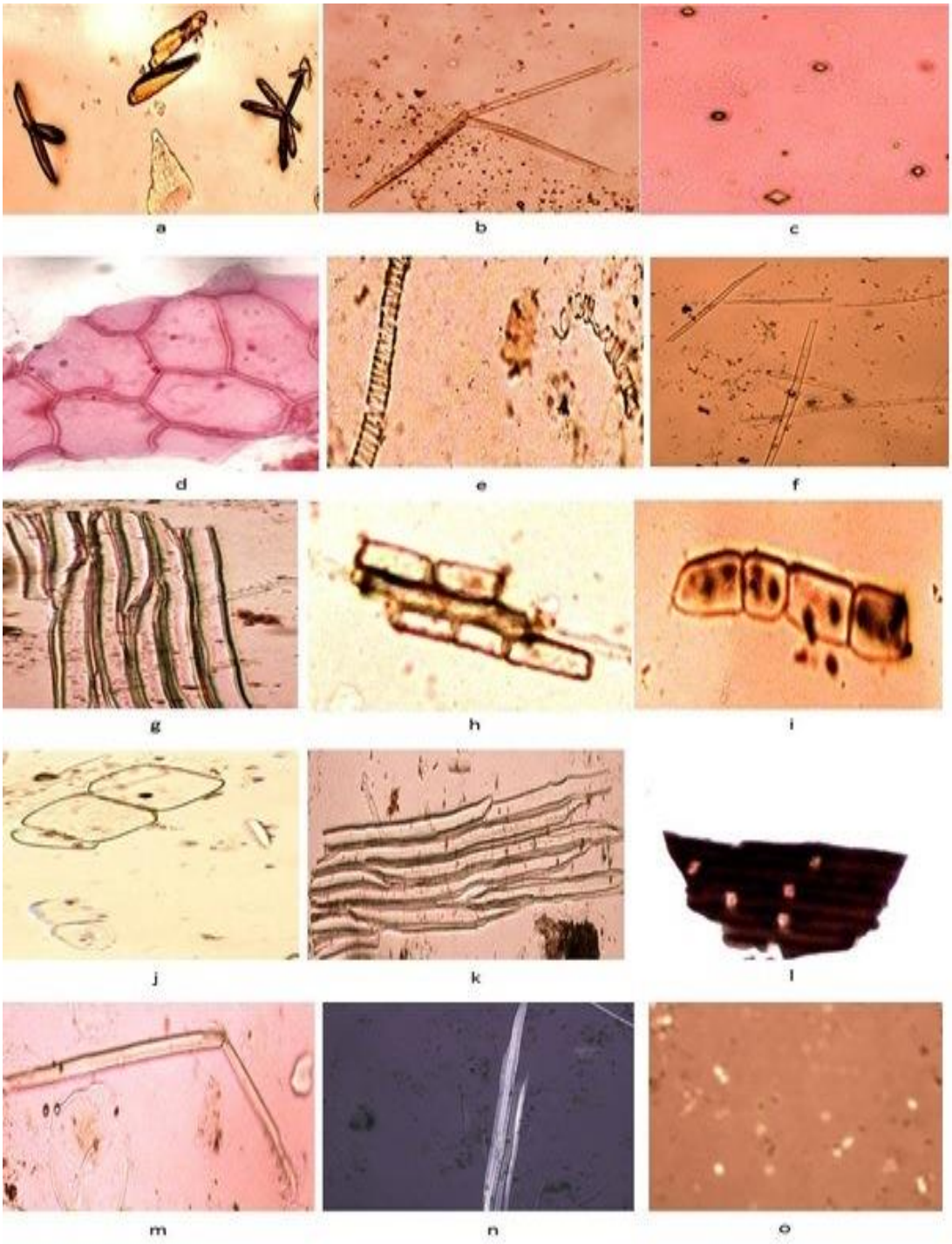


Figure 10: Powder characteristics a) Astrosclereides, b) Needle shaped raphides, c) Tetragonal calcium oxalate crystals, d) Epidermal cells, e) spiral thickening of xylem vessels, f) narrow fibres, g) Parenchyma in cortex, h) parenchyma cell, i) sclereids, j) lignified pitted parenchyma, k) fibres and sclereids, l) cork cells, m) septate fibre, n) phloem fibre, o) starch grain.

2.3. Quantitative microscopy

Quantitative microscopy is useful for fixing standards for crude drugs. Values of different leaf constants are as follows Stomatal number (upper and lower) was 140-180 per mm^2 and 150-250 per mm^2 respectively, Stomatal index (upper and lower) was 15.5-18.5 and 21.5- 25.5 respectively, Vein islet number was 8.6-12.8 per mm^2 and Vein termination number was 9.2-13.1 per mm^2. The results are shown in table 7.

Table 7: Quantitative microscopical parameters

s.no.	Parameter	Range
1	Stomatal number (upper)	140-180 per mm^2
2	Stomatal number (lower)	150-250 per mm^2
3	Stomatal index (upper)	15.5-18.5
4	Stomatal index (lower)	21.5-25.5
5	Veinislet number	8.6- 12.8
6	Vein termination number	9.2- 13.1
7	Palisade ratio	1.12-1.15

3. Random amplified polymorphic DNA (RAPD) fingerprinting profile

The genomic DNA isolated from *Exacum lawii* and purity was A_{260}/A_{280} ratio was found to be 1.6. The DNA was subjected to gel electrophoresis and image was shown if figure 1. The 6 primers pairs were successfully used for amplification of *matK* and *rbcL* gene. The primer pair rbcL_1F & rbcL_724R, rbcLaF & rbcLarR and MatK_2.1F & MatK_5R produced two DNA fragments from 700 to 800 bp (Figure 11).

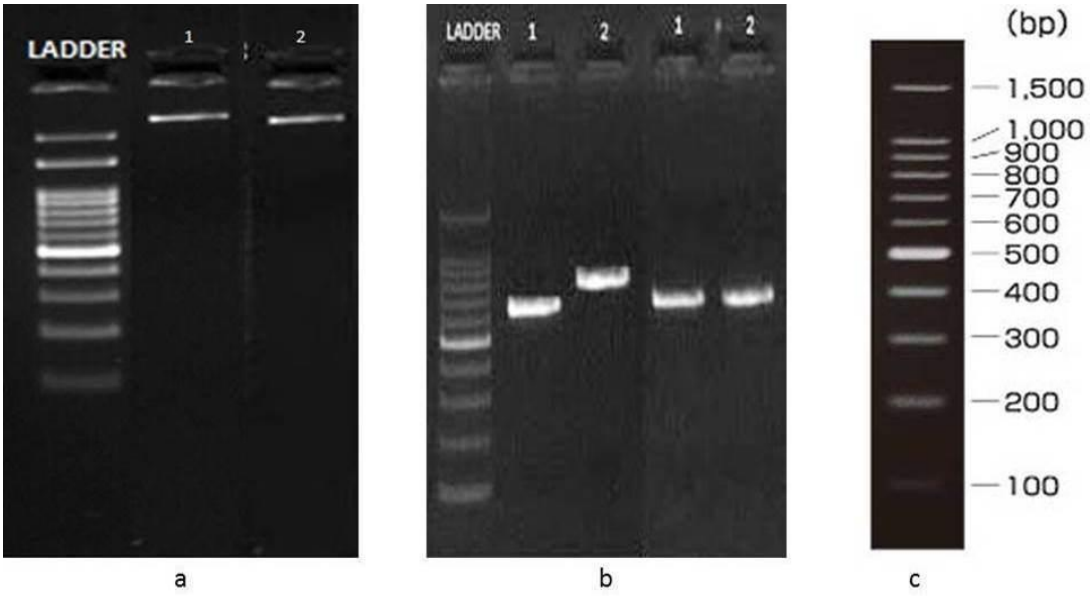


Figure 11: a) Gel electrophoretogram of DNA isolated from *Exacum lawii* b) The obtained RAPD-PCR products for *Exacum lawii* showing bands of PCR amplified products of matK and rbcL universal primers. C) Specifications of ladder

4. Nutritional content analysis and physicochemical Evaluation

The whole plant of *Exacum lawii* was dried in shade at room temperature for analysis of vitamins. The results were expressed as mg of element per 100 gm of dry weight plant material. From the results, vitamin C was found to be in higher amount 6.11 mg/100 gm as ascorbic acid. The calcium content (12871.67 mg/kg) was found to be in appreciative quantitative in comparison with other macroelements. It showed *Exacum lawii* can be a good source of calcium. Fatty acid profile showed different concentration of saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans-fatty acids. Palmitic acid (1.37 gm/100 gm), Stearic acid (0.41 gm/100 gm), Oleic acid (0.41 gm/100 gm), Linoleic acid (1.11 gm/100 gm) and alpha-Linolenic acid (0.76 gm/100 gm) along with other fatty acids. Energy in calories was estimated to be 44.73 kcal/100 gm (Table 8).

Determination of crude fiber content (Dutch method)

The crude fibre content was found to be 34.9% w/w. The results of the quantitative physicochemical constants of the air dried powder material were represented in table 9.

Heavy metal estimation

Heavy metals content (in PPM) were calculated and given in table 9. The values were found to be within the limit as prescribed by the WHO guidelines.

Fluorescence powder drug analysis

Fluorescence analysis shows the colour emitted by the powdered drug it is important procedure for testing purity of drug. Results are shown in table 10.

5. Pesticide residue

The chlorinated pesticide present in the whole plant of Exacum lawii in first and the second elute was reported to be 0.023±0.001 mg/kg and 0.012±0.00 mg/kg of plant material. The phosphate pesticide from the first and second elute of the column was found to be 0.021±0.001 mg/kg and 0.018±0.001 mg/kg of plant material respectively. While in third elute pesticide content was reported to be absent.

Table 8: Nutritional content analysis

Vitamins	Methods	Results
Vitamin E	AOAC 2001.13	5.06 mg/100 g
Vitamin A	AOAC 2001.13	<100.0 IU/100 g
Vitamin C	AOAC 2012.21	6.11 mg/100 g
Minerals		
Calcium (Ca)	AOAC2011.14	12871.67 mg/kg
Potassium (K)	AOAC2011.14	8041.58 mg/kg
Iron (Fe)	AOAC2011.14	86.15 mg/kg
Sodium (Na)	AOAC2011.14	6196.54 mg/kg
Fatty acid profile		
Monounsaturated fatty acids (total)	AOAC 996.01	1.21 g/100 g
Polyunsaturated fatty acids (total)	AOAC 996.01	1.19 g/100 g
Tans-fatty acids (total)	AOAC 996.01	<0.1 g/100 g
Fatty acid composition	AOAC 996.01	
Margaroleic acid		<0.1 g/100 g
homo-gamma-Linolenic		<0.1 g/100 g
Caproic acid		<0.1 g/100 g
Caprylic acid		<0.1 g/100 g
Capric acid		<0.1 g/100 g
Undecanoic acid		<0.1 g/100 g
Lauric acid		<0.1 g/100 g
Tridecanoic acid		<0.1 g/100 g
Myristic acid		<0.1 g/100 g
Myristoleic acid		<0.1 g/100 g
Pentadecanoic acid		<0.1 g/100 g
Pentadecanoic acid + Isomers		<0.1 g/100 g
Palmitic acid		1.37 g/100 g
Palmitoleic acid		<0.1 g/100 g
Margaric acid		<0.1 g/100 g
Stearic acid		0.41g/100 g
Oleic acid		0.41 g/100 g
Linoleic acid		1.11 g/100 g
Linolelaidic acid		<0.1 g/100 g
alpha-Linolenic acid		0.76 g/100 g
Arachidic acid		<0.1 g/100 g
Eicosenoic acid		<0.1 g/100 g
Eicosadienoic acid		<0.1 g/100 g
Eicosatrienoic acid		<0.1 g/100 g
Aracidonic acid		<0.1 g/100 g
Eicosapentaenic acid		<0.1 g/100 g
Heneicosanoic acid		<0.1 g/100 g
Behenic acid		<0.1 g/100 g
Docosenoic acid + isomers		<0.1 g/100 g
Docosadienoic acid		<0.1 g/100 g
Docosahexanoic acid		<0.1 g/100 g
Tricosanoic acid		<0.1 g/100 g
Lignoceric acid		<0.1 g/100 g
Nervonic acid		<0.1 g/100 g
Elaidic acid		<0.1 g/100 g
Gamma-Linolenic acid		<0.1 g/100 g

Table 9: Physicochemical parameters of *Exacum lawii*

S.No.	Quantitative standards			Observation
1	*Moisture content			6.10% w/w
2	*Swelling index			0.89 % w/v
3	Foaming index			< 100
4	*Total ash			6.06% w/w
5	*Acid insoluble ash			1.26% w/w
6	*Water soluble ash			2.49% w/w
7	*Bulk density			0.59% w/w
8	*Foreign matter			< 0.5% w/w
9	Volatile oil content			0.10% yield (ml/100 gm of dry weight)
10	*Crude fibre content			34.9% w/w
11	Heavy metal content	λ_{max}	Fuel flowrate(l/min)	Concentrations in PPM
	Lead (Pb)	282.3	2.0	Not more than 0.010 PPM
	Cadmium (Cd)	228.7	1.8	Not more than 0.0002 PPM
	Zinc (Zn)	211.9	2.0	Not more than 0.235 PPM
	Mercury (Hg)	253.5	1.8	Not more than 0.220 PPM

*** Values are mean ± SEM, (n=3)**

Table 10: Fluorescence analysis of plant powder

S. No	Powder + reagent	Fluorescence in day light	Fluorescence (254 nm)	Fluorescence (365 nm)
1	Powder as such	Green	Green	Green
2	Powder + 1 N NaOH in methanol	Yellow green	Light green	Dark green
3	Powder+1 N NaOH in water	Yellow green	Light green	Dark green
4	Powder +1 N HCl in methanol	Yellowish green	Light green	Brown
5	Powder + 1 N HCl in water	Yellowish green	Light green	Brown
6	Powder + 1N HNO_3 in methanol	Brownish orange	Yellowish green	Dark green
7	Powder+1N HNO_3 in water	Light brown	Yellowish green	Dark green
8	Powder + Iodine (5%)	Dark green	Dark green	Blackish green
9	Powder + $FeCl_3$ (5%)	Yellowish green	Dark green	Brown
10	Powder + KOH (50%)	Dark brown	Yellowish brown	Brown
11	Powder + ammonia (25%)	Light green	Yellowish green	Dark green
12	Powder + H_2SO_4	Dark brown	Dark brown	Black
13	Powder + saturated picric acid	Greenish yellow	Light yellow	Light yellow
14	Powder + acetic acid	Greenish yellow	Greenish yellow	Light yellow

6. *In vitro* antioxidant activity

The total antioxidant capacity of ELE and its various fractions, ethyl acetate fraction (EAE), chloroform fraction (CHE), aqueous fraction (AQE) and hexane fraction (HE) was determined by using the linear regression equation of the calibration curve and was expressed as the equivalent number of ascorbic acid. Among the tested fractions, ELE depicted the highest antioxidant capacity (90.17±1.65 µg/ml ascorbic acid equivalent) than the other fractions i.e. EAE: 81.1±1.08 µg /ml, CHE: 63.84±1.3 µg /ml, AQE: 52.34±1.16 µg /ml and HE: 25.5 ± 0.52 µg/ml ascorbic acid equivalent respectively. The study depicted that reducing power of the tested extract and its fractions is concentration dependent i.e. ELE: 0.364 ± 0.004, EAE: 0.246 ± 0.006 µg /ml, CHE: 0.23 ± 0.009 µg /ml, AQE: 0.186 ± 0.003 µg /ml and HE: 0.023 ± 0.002 µg/ml ascorbic acid equivalent respectively.

All the fractions tested in the present study had considerable DPPH free radical scavenging activity and indicated by measured IC_{50} values. Standard ascorbic acid was found to have lowest IC_{50} value of 73.45±1.26 µg/ml followed by ELE (IC_{50}: 117.55±2.26 µg/ml), EAE (IC_{50}: 136.35±0.49 µg/ml), CHE (IC_{50}: 152.32±0.37 µg/ml), AQE (IC_{50}: 142.15±0.67 µg/ml) and HE (IC_{50}: 156.24 ± 0.74 µg/ml).

From the results, it was found that ELE and its fractions were capable of scavenging hydrogen peroxide in a concentration dependent manner. Rutin depicted the highest scavenging activity with an IC_{50} value of 69.65±0.72 µg/ml followed by ELE (IC_{50}: 87.68±0.61 µg/ml), EAE (IC_{50}: 90.9±0.49 µg/ml), CHE (IC_{50}: 206.14±0.38 µg/ml), AQE (IC_{50}: 221.82±0.44 µg/ml) and HE (IC_{50}: 216.19 ± 0.19 µg/ml).

Rutin is considered to be highly potent nitric oxide scavenging activity (IC_{50} value 74.92±2.4 µg/ml). ELE (IC_{50}: 82.38±0.61 µg/ml) followed with EAE, AQE and CHE have IC_{50} value of 88.78±2.2 µg/ml, 234.73±1.87 µg/ml and 242.85±2.84 µg/ml respectively, whereas HE showed the least scavenging activity (IC_{50} value 242.84±2.8 µg/ml).

Iron (II)–dependent deoxyribose damage assay was used for determining the effect of on inhibition potential of extract and its fraction on hydroxyl radical production. Hydroxyl radical scavenging activity was performed and results revealed that standard Butylated hydroxyanisole (BHA) (IC_{50}: 64.86±0.36 µg/ml) showed maximum activity which was followed by ELE (IC_{50}: 82.42±0.72 µg/ml), EAE (IC50: 124.68±0.56 µg/ml), CHE (IC_{50}: 160.52±0.54 µg/ml), AQE (IC_{50}: 168.23±0.37) whereas HE (IC_{50}:183.58±0.68) has least scavenging activity. The percentage inhibition of ELE and its fractions were obtained from various methods are shown in figure 12. The ethanolic extract of *Exacum lawii* was found to be more potent antioxidant and comparable to standard drugs respectively.

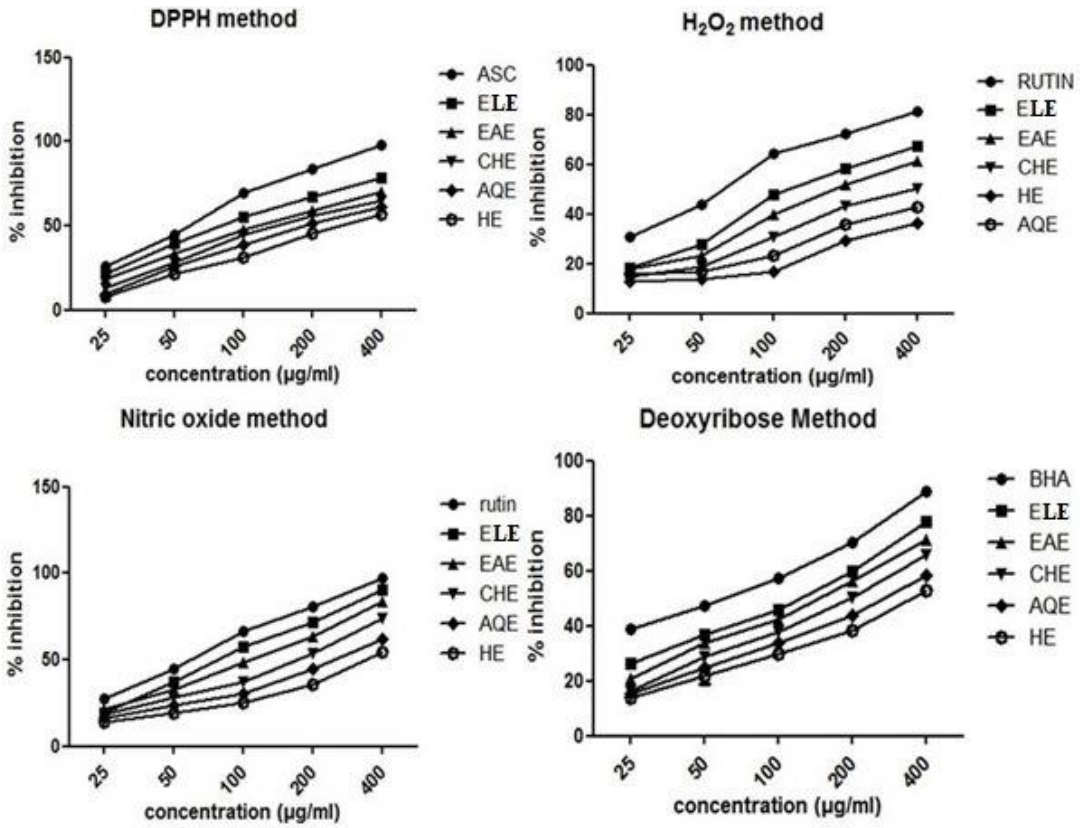


Figure 12: In vitro antioxidant activity of ELE and its various fractions. Where, Asc: Ascorbic acid, BHA: Butylated Hydroxy Anisole, ELE: Ethanolic extract of *Exacum lawii*, EAE: Ethyl acetate fraction of *Exacum lawii*, CHE: Chloroform fraction of *Exacum lawii*, AQE: Aqueous fraction of *Exacum lawii*, HE: Hexane fraction of *Exacum lawii*.

PHYTOCHEMICAL EVALUATIONS

1. Preliminary Phytochemical screening

The phytochemical investigation indicated the presence of alkaloids, flavonoids, phenolic compounds, terpenoids, steroids and coumarins within ethanolic extract and its toluene fraction, chloroform fraction, ethylacetate fraction and tannins are found to be present in ethanolic extract and aqueous fraction (Table 11).

Table 11: Preliminary phytochemical screening of different *Exacum lawii* extracts

Phytoconstituents	Ethanolic extract	ethyl acetate fraction	chloroform fraction	Aqueous fraction	Petroleum ether fraction
Alkaloids	+	+	+	+	-
Glycosides	+	+	-	-	+
Flavonoids	+	+	+	+	-
Steroids	+	+	+	+	+
Phenols	+	+	+	+	-
Saponins	+	-	-	-	-
Proteins	-	-	-	-	-
Amino acids	+	-	-	-	-
Coumarin	+	+	-	+	-
Tannin	-	-	-	-	-
Terpenoid	+	+	+	+	-
Reducing sugar	-	-	-	-	+

(+) indicate presence, (–) indicate absence.

2. Quantitative estimation

The study found that ethanolic extract contains quantitatively more phytoconstituents when compared with different fractions. The total phenolic content, Flavonoid content, Flavonol content and Alkaloidal content of ELE and its different fractions are given in table 12.

Table 12: Quantitative estimation of various classes of phytochemical compound

Extracts	Extractive value (%w/w)	Phenolic content (mg GAE /gm)	Flavonoid content (mg rutin/gm)	Flavonol content (mg rutin/gm)	Alkaloidal content (mg/gm)
Ethanolic extract	10.70% w/w	12.21±0.58	21.86±0.22	15.53±0.05	35.59±0.13
Aqueous fraction	12.65% w/w	8.34±0.16	9.33±0.08	10.1±0.32	15.5±0.12
Chloroform fraction	4.56% w/w	6.82±0.16	18.29±0.07	8.46±0.2	12.39±0.15
Ethyl acetate fraction	1.98% w/w	8.33±0.10	6.55±0.12	5.5±0.10	26.29±0.16
Petroleum ether fraction	8.48% w/w	1.45±0.15	1.56±0.10	0.91±0.02	6.53±0.15

Each column represents as means ± SD (n = 6)

3. Thin Layer chromatography

The results obtained from TLC analysis suggest that ELE and its fractions contain alkaloids, phenols, flavonoids, terpenoids and steroids. Results of TLC analysis in different solvent system of ELE and its fractions with Rf value for particular phytocompound is given in table 13.

Table 13: TLC analysis of ethanolic extract of *Exacum lawii* and its fraction

Phytocompound	**Solvent system**	**Rf value**	**fraction**
Alkaloids	Toluene: ethylacetate: diethylamine (70:20:10)	0.15-0.45	Toluene fraction Chloroform fraction Ethyl acetate fraction
Terpenoids	Ethylacetate: methanol: water (77:15:8)	0.3-0.8	Ethanolic extract Aqueous fraction Ethyl acetate fraction
Flavonoid	Ethylacetate: formic acid: glacial acetic acid: water (100:11:11:27)	0.45-0.65	Ethanolic extract Chloroform fraction Ethyl acetate fraction Aqueous fraction
phenols	chloroform: ethylacetate: formic acid (5:4:1)	0.1-0.42	Ethanolic extract Aqueous fraction
Steroids	Toluene: ethylacetate (9.3:7)	0.75-0.8	Ethanolic extract Chloroform fraction Ethyl acetate fraction Aqueous fraction Toluene fraction

4. HPLC standardization with chemotaxonomic marker swertiamerin

Chromatograms of ELE along with standard swertiamarin have been shown in figure 13. Mean retention time was of 1.3 minutes 16 minutes. The concentration of swertiamarin in ELE was found to be 119.59 µg/gm of *Exacum lawii* extract.

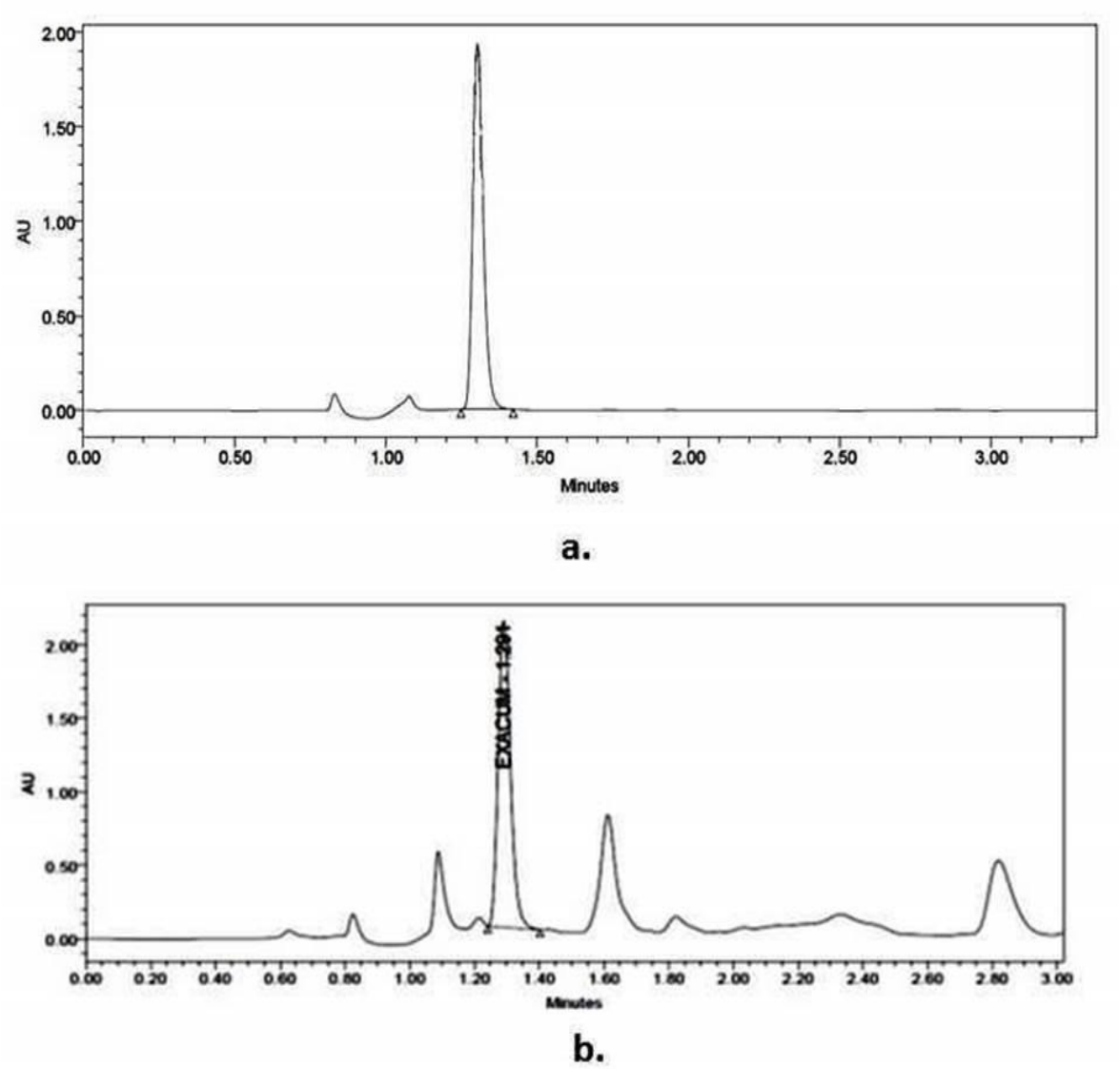


Figure 13: a) HPLC chromatogram showing the peak of standard Swertiamerin, b) HPLC chromatogram of ethanolic extract of *Exacum lawii* showing peak of Swertiamerin.

5. GCMS analysis of *Exacum lawii* extract

GC-MS analysis of ELE led to the identification of 20 compounds. The chromatogram is shown in figure 14 and identified compounds were listed in the table along with retention time, peak area, peak percentage area, structure and molecular weight (Table 14).

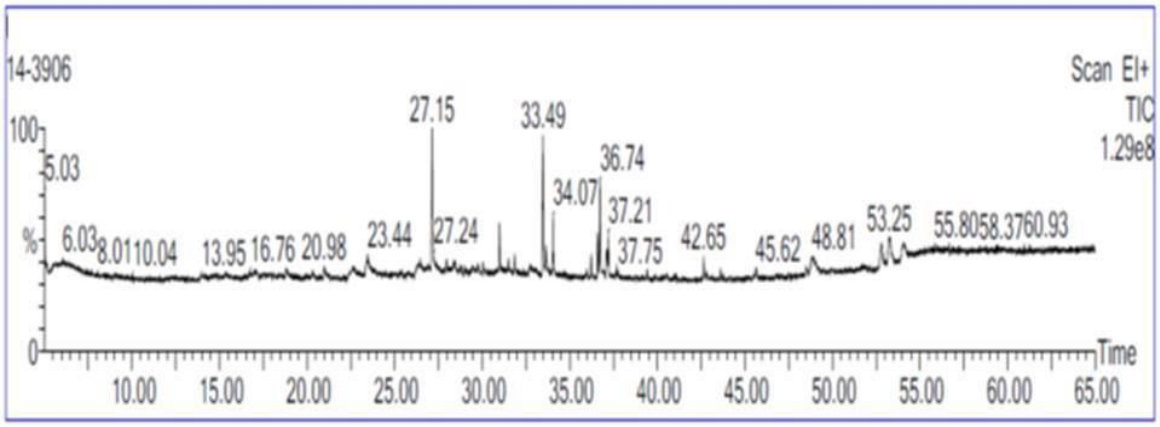
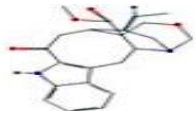
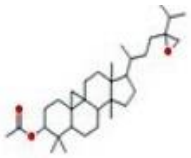
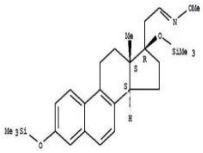
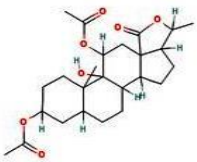
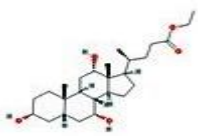
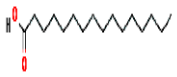
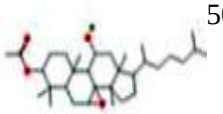
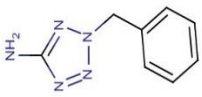
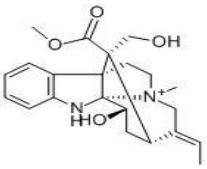
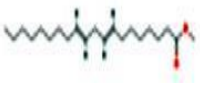
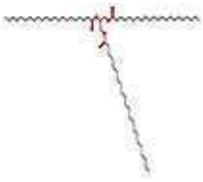
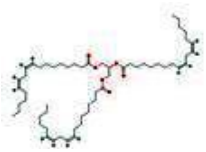


Figure 14: GC-MS chromatogram of ethanolic extract of *Exacum lawii*

Table 14: Some compounds identified by GCMS in ethanolic extract of *Exacum lawii*

S.No	Compound	RT	Area	% area	Mol. form.	Structure	Mol.wt. (gm/mol)
1	Pagicerine	5.61	149241	0.50	$C_{22}H_{29}N_2O_4$		382
2	9,19-Cyclolanostan-3-ol, 24,24-epoxymethano-, acetate	5.85	93391	0.31	$C_{33}H_{54}O_3$		498
3	19-Norpregna-1,3,5,7,9-pentaen-21-al, 3,17-bis[(trimethylsilyl)oxy]-, O-methyloxime, (17à)-	6.052	176169	0.59	$C_{27}H_{41}NO_3Si_2$		490
4	pregnan-18-oic acid, 3,9,11,20-tetrol, 3,11-diacetate, 18,20-lactone	6.38	44109	0.15	$C_{25}H_{36}O_7$		448

6	Ethyl iso-allocholate	22.63	544585	1.84	$C_{26}H_{44}O_5$		418
7	n-Hexadecanoic acid (Palmitic acid)	33.49	4241966	14.31	$C_{36}H_{58}O_6$		256
8	7,8-Epoxylanostan-11-ol, 3-acetoxy-	26.35	145521	0.49	$C_{32}H_{54}O_4$		502
10	Phenylalanine, 4-amino-N-t-butyloxycarbonyl-, t-butyl ester	26.91	112566	0.38	$C_{18}H_{28}N_2O_{412}$		336
11	2H-Tetrazol-5-amine, 2-(phenylmethyl)-	27.15	5429788	18.31	$C_8H_9N_5$		175
12	Echitamine	28.43	308625	1.04	$C_{22}H_{29}N_2O_4$		384
13	8,11-Eicosadienoic acid, methyl ester	31.01	1278947	4.31	$C_{21}H_{38}O_2$		323
14	Ascorbic acid 2,6-dihexadecanoate	33.49	4241966	14.31	$C_{38}H_{68}O_8$		652
15	Dodecanoic acid, ethyl ester	34.07	1463905	4.94	$C_{14}H_{28}O_2$		228
16	Docosanoic acid, 1,2,3-propanetriyl ester	45.679	360115	1.22	$C_{69}H_{134}O_6$		689
17	Trilinolein	36.61	751095	2.53	$C_{57}H_{98}O_6$		878
20	Astaxanthin	52.8	2125073	7.17	$C_{40}H_{52}O_4$		596

6. Isolation and identification of Swertiamerin

The Swertiamerin was isolated by using column chromatography and yield obtained was 22.54 mg in 500gm of dried powder of *Exacum lawii*. Characterization was carried by measuring melting point which is found same as swertiamerin that is 113°C to 114°C. UV absorption maxima (λ max) calculated was to be 238 nm in methanol. Structure of isolated swertiamerin was identified by infrared spectrum reflects 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, 1282.71 cm^{-1} for C-O stretching (Figure 15-16).

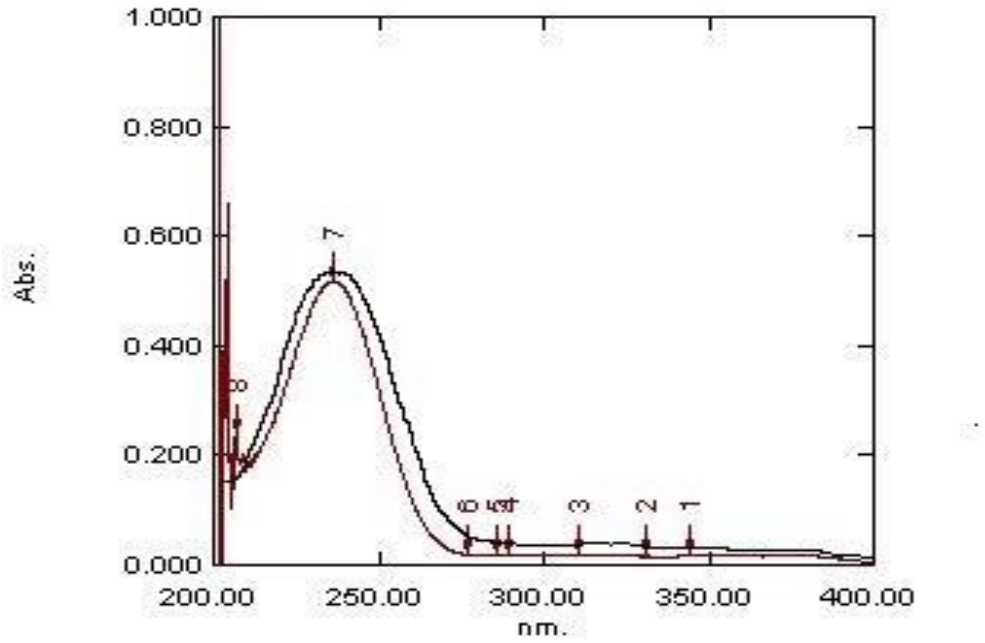


Figure 15: Overlay of ultraviolet absorption spectrum of swertiamarin isolated in lab and reference standard

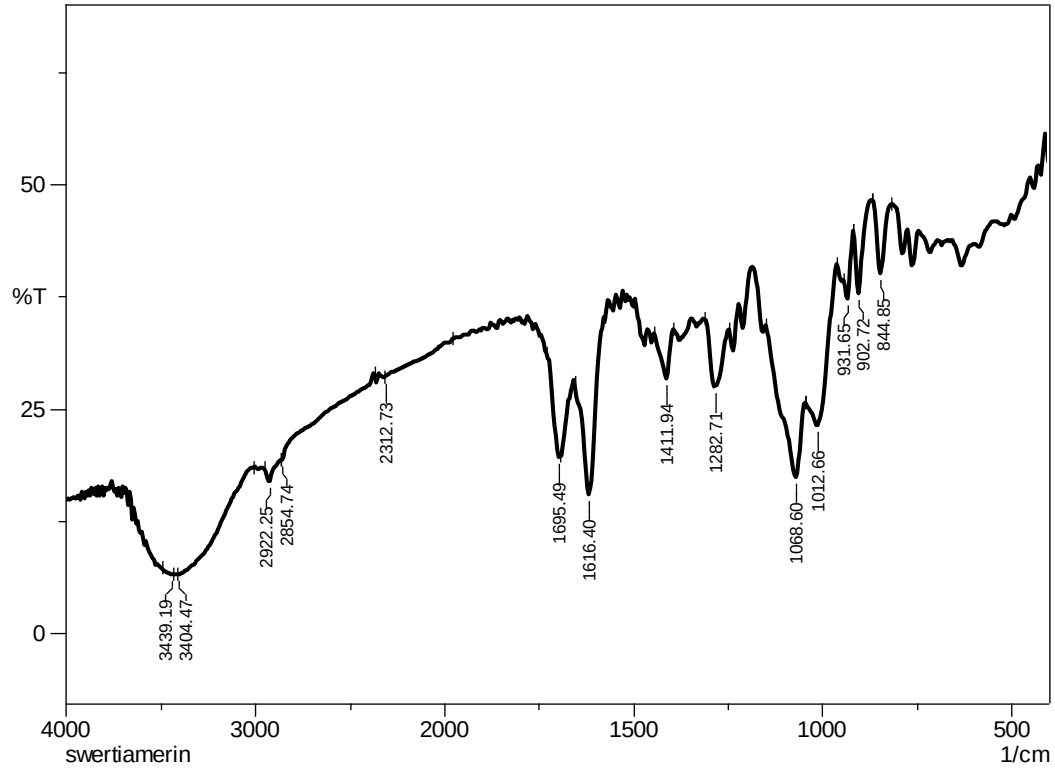


Figure 16: FTIR spectra of isolated swertiamerin

6.1. Structure elucidation of swertiamarin

^{1}H NMR (500 MHz, DMSO-d6) δ (ppm): 7.513 (s, 1H, C8-pyranopyrone), 5.592 (s, 1H, OH-pyranopyrone), 5.393-5.368 (dd, 2H, C2-vinyl), 5.291(s, 1H, OH-C3-tetrahydropyran), 5.261-5.236 (m, 1H, C1-vinyl), 5.019, 5.009 (d, 1H, C6-pyranopyrone), 4.984, 4.973 (d, 1H, C2-tetrahydropyran), 4.711 (s, 1H, OH-C4-tetrahydropyran), 4.615-4.564 (t, 2H, C3- pyranopyrone), 4.465, 4.450 (d, 1H, C5-pyranopyrone), 4.262 (s, 1H, OH-C5- tetrahydropyran), 3.701-3.667 (t, 1H, C3-tetrahydropyran), 3.465-3.418 (t, 1H, C4- tetrahydropyran), 3.198,3.153 (d, 2H, CH_2-hydroxymethy), 3.078-3.030 (t, 1H, C5- tetrahydropyran), 3.010-2966 (m, 1H, C6-tetrahydropyran), 2.828 (s, 1H, OH-hdroxymethy), 1.770-1.683 (t, 2H, C4-pyranopyrone) (Figure 17).

^{13}C NMR (500 MHz, DMSO-d6) δ (ppm): 164.413 (C=O), 151.963 (C8-pyranopyrone), 132.893 (C1-vinyl), 120.362 (C2-vinyl), 108.134 (fused carbon-pyranopyrone), 98.288 (C2-tetrahydropyran), 96.455 (C6- pyranopyrone), 77.449 (C6-tetrahydropyran), 76.079 (C4-tetrahydropyran), 72.882 (C3-tetrahydropyran), 69.968 (C5-tetrahydropyran), 64.116 (C3- pyranopyrone), 62.426 (fused carbon-pyranopyrone), 60.913 (hydroxymethyl), 40.009 (C5, pyranopyrone), 32.075 (C4-pyranopyrone) (Figure 18).

ES-IMS (*m*/*z*): Molecular weight was found to be 374 .34, by analysing peaks forming the adduct with Na^+, 397.50 (M+Na, 30%) and 771.27 (2M+Na, 100%). (Figure 19)

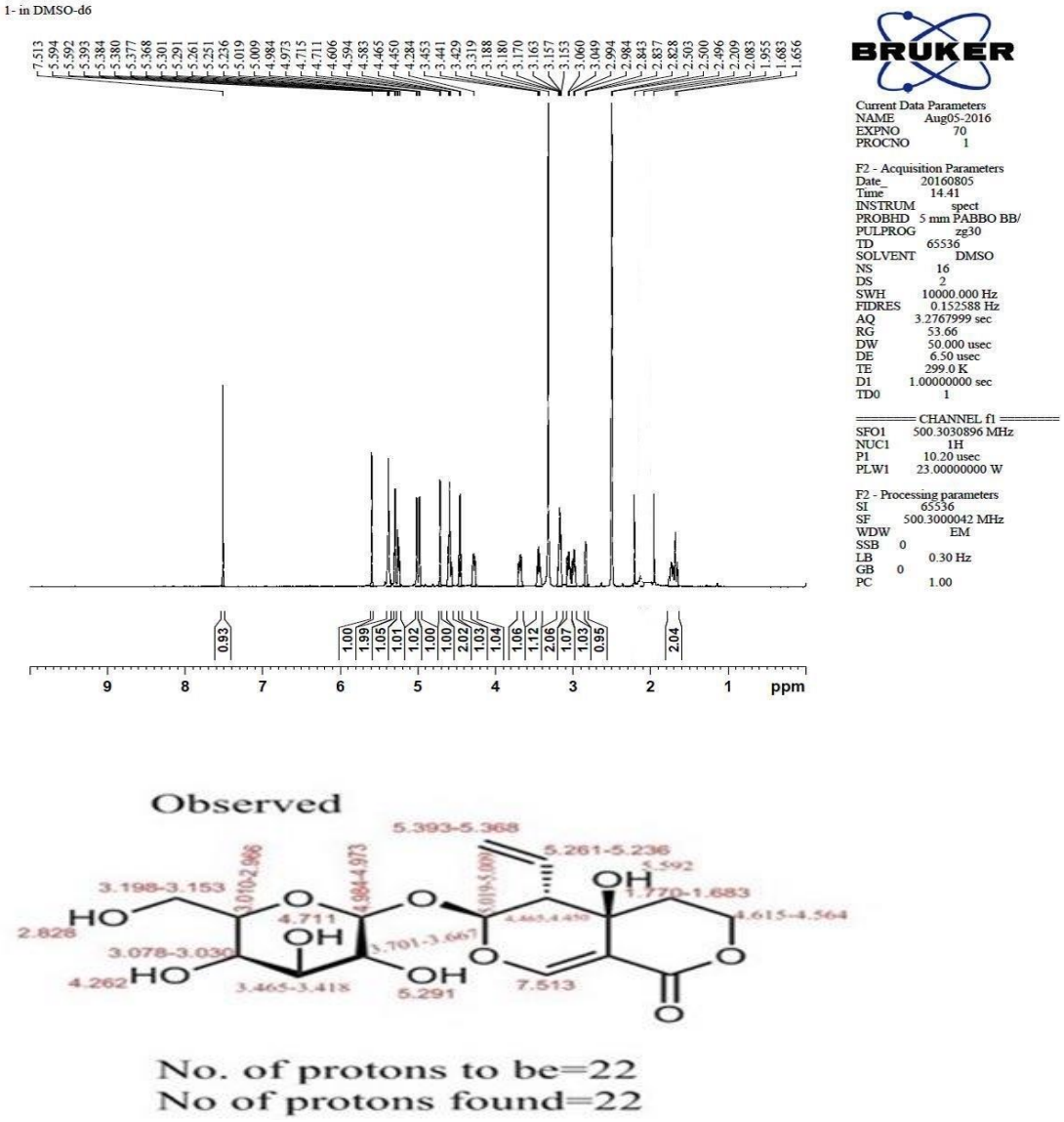


Figure 17: [1]H NMR spectra of isolated swertiamerin.

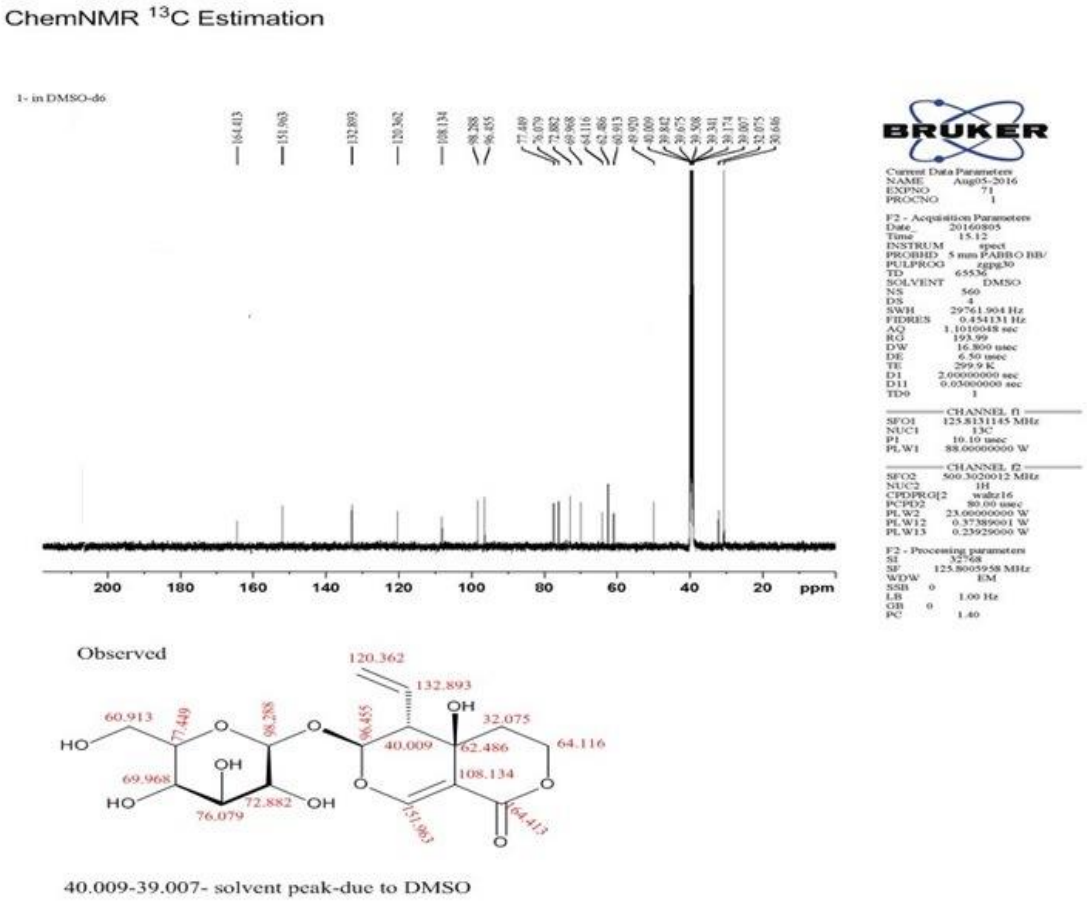


Figure 18: ^{13}C NMR spectra of isolated swertiamerin**.**

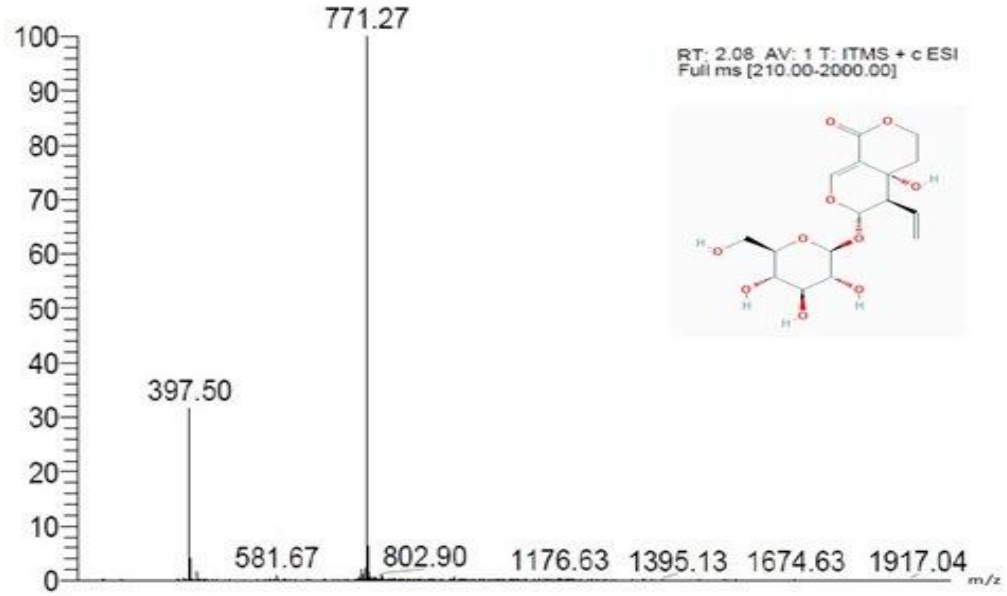


Figure 19: LCMS spectra isolated swertiamerin.

7. Molecular docking study

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 calculated K_i values and the experimental Growth percentage values are given in Table 2. 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 with in 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 (Figure 20-22) (Table 15).

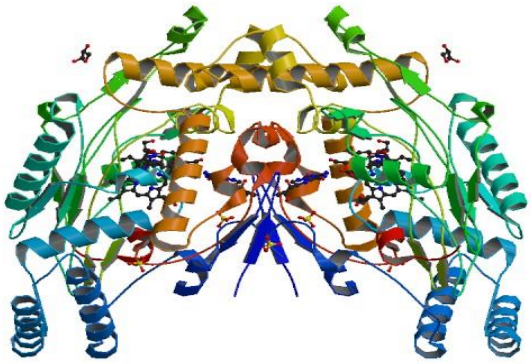


Figure 20: Structure of NOS heme domain

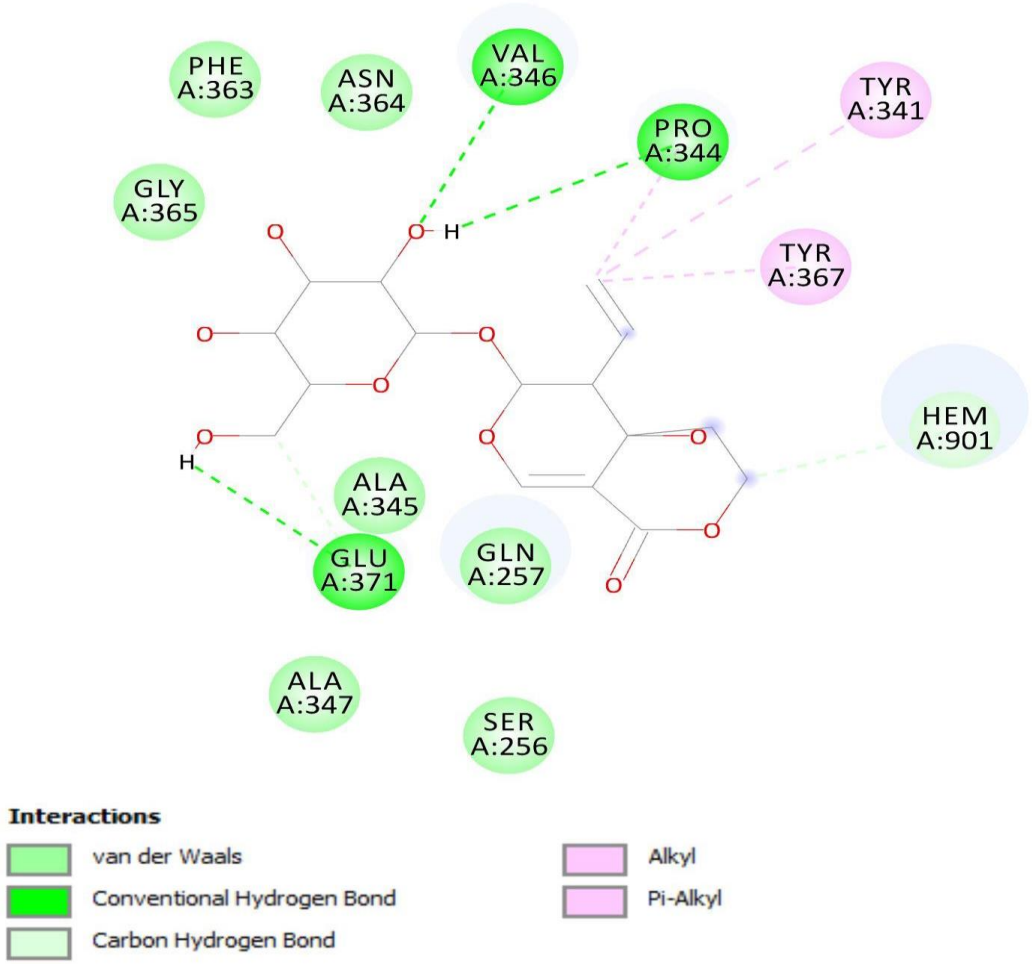


Figure 21: Swertiamarin-3NQS docking showing different bonds

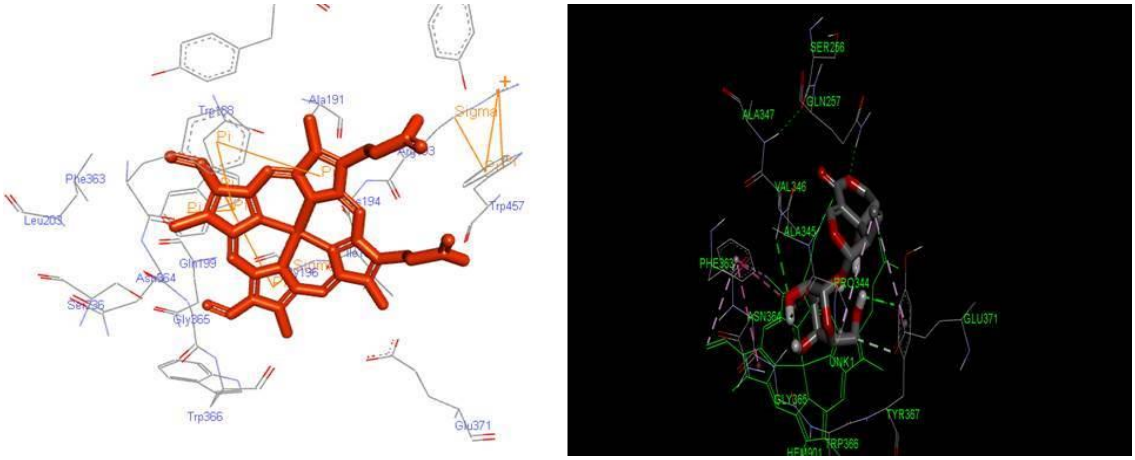


Figure 22: Swertiamarin-3NQS docking shows different predicted bonding interactions.

Table 15: Interactions obtained in 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

PHARMACOLOGICAL EVALUATION

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

1.1. Toxicity profile of ELE

1.1.1. Acute oral toxicity study for ELE

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

1.1.2. Repeated dose 28-days oral toxicity study for ELE

Preclinical signs

Throughout the period of 28 days of study, there were no clinical signs like tremors, convulsions, loss of consciousness, *irregular breathing*, aggression, lethargy, diaphragmatic breathing, gait, piloerection and licking were observed. Study indicates that the limit of dose that may result in mortality was higher than 2000 mg/kg, p.o.

Effects on body weight and organ weight

Change in body weight and organ weight of control and ELE treated rats are presented in (Figure 23) and (Table 16), respectively. The result showed that animals of each group of the study survived with a normal weight gain pattern until the termination of experiment. ELE (1000 mg/kg, p.o., 2000 mg/kg, p.o. and 4000 mg/kg, p.o. per day) administered for 28 days induced no significant differences in vital organ

weight when compared with the control group. The satellite group showed no significant increase in the vital organ weight.

Effects on haematological and biochemical parameters

Rats treated with various doses revealed no significant difference when compared with the normal control. All parameters were under the standard range (Table 17-18).

Histopathological examination

Rats treated with ELE showed normal structure, shape, size, texture and colour of vital organs (kidneys, livers, stomach, heart, pancreas, brain and lungs). Transverse section of vital organs were observed using the light microscopy examinations showed normal histology in organs, absence of any gross pathological lesion, sign of inflammation and vacuolisation after comparison with normal control group (Figure 24).

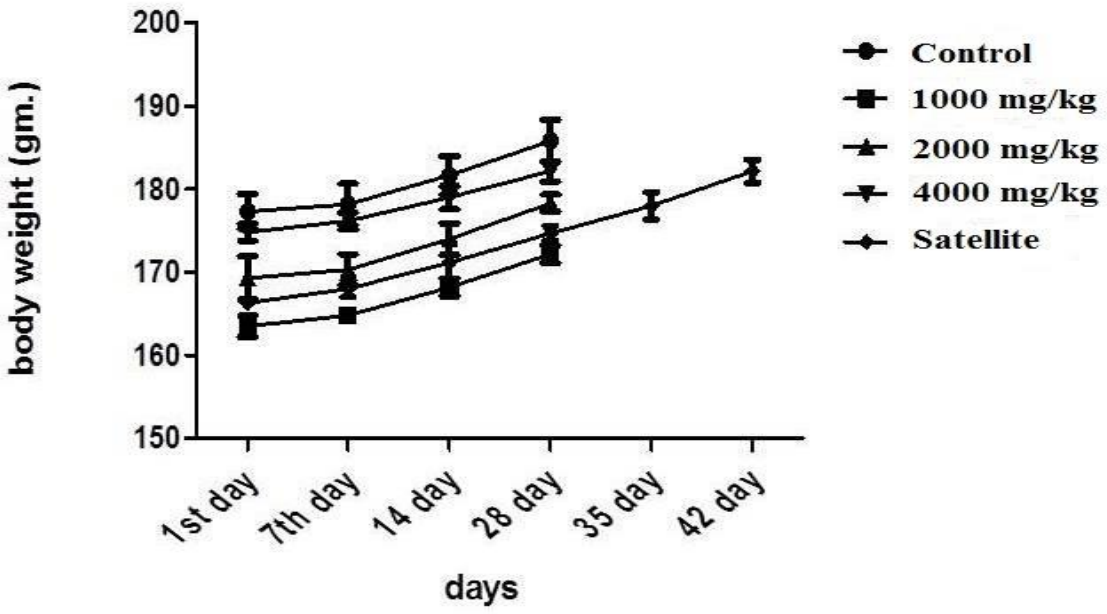


Figure 23: Change in body weight during oral toxicity study

Table 16: Effect of different doses of ELE on vital organ weight during oral toxicity study

	***Exacum lawii* ethanolic extract (Dose)**				
Organ (gm.)	**Control**	**1000 mg/kg**	**2000 mg/kg**	**4000 mg/kg**	**Satellite**
Heart	1.03±0.14	0.995±0.13	1.13±0.05	1.18±0.037	1.09±0.04
Brain	1.98±0.04	2.04±0.06	2.14±0.08	2.02±0.05	2.23±0.14
Liver	8.68±0.15	9.15±0.18	9.07±0.14	9.28±0.14	9.06±0.13
Kidney rt.	3.18±0.09	3.39±0.07	3.47±0.11	3.63±0.063	3.39±0.13
Kidney lt.	2.5±0.11	2.48±0.09	2.36±0.08	2.67±0.11	2.59±0.08
Spleen	1.42±0.19	1.56±0.07	1.50±0.06	1.46±0.08	1.52±0.11
Stomach	1.64±0.032	1.65±0.03	1.76±0.03	1.51±0.03	1.65±0.08

Values are mean ± SEM, (n=10). One way ANOVA followed by Newman–Keuls Multiple Comparison test, *P value <0.05, **P value <0.01, ***P value <0.001.

Table 17: Effect of different doses of ELE on Biochemical parameters during oral toxicity study

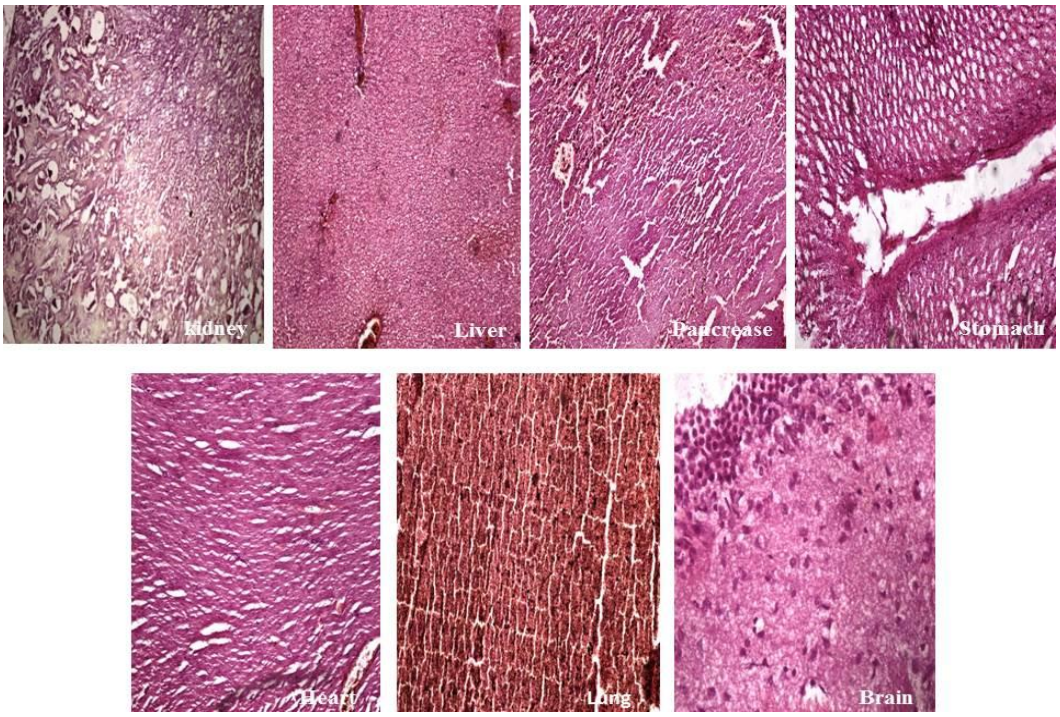
		***Exacum lawii* ethanolic extract (Dose)**				
Biochemical parameters	**units**	**Control**	**1000 mg/kg**	**2000 mg/kg**	**4000 mg/kg**	**Satellite**
total protein	(gm/dl)	7.5±0.16	6.85±0.15	8.3±0.45	7.89±0.12	8.1±0.11
SGOT	(U/l)	151.9±5.6	150.86±4.25	148.73±2.8	153.66±4.7	155.7±3.3
SGPT	(U/l)	76.03±2.6	73.6±1.07	79.67±2.78	76.6±4.5	77.75±2.6
urea	(mg/dl)	50.7±1.8	52.4±3.3	49.96±1.4	53.03±2.7	55.65±3.7
creatinine	(mg/dl)	0.37±0.01	0.35±0.01	0.36±0.01	0.41±0.012	0.45±0.02

Values are mean ± SEM, (n=10). One way ANOVA followed by Newman–Keuls Multiple Comparison test, *P value <0.05, **P value <0.01, ***P value <0.001.

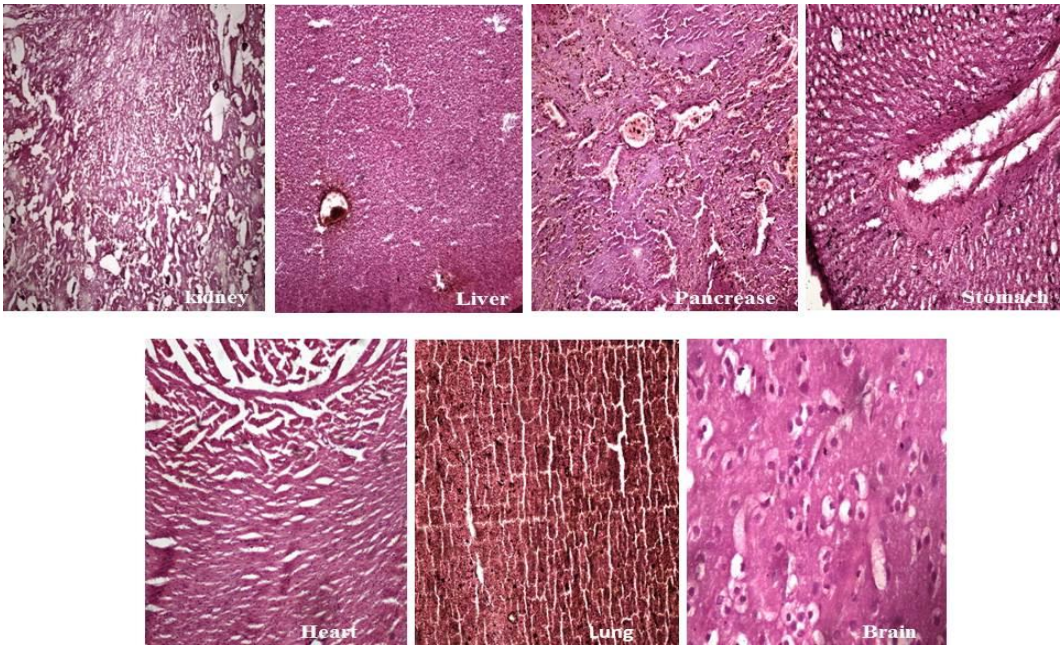
Table 18: Effect of different doses of ELE on Haematological parameters during sub-acute toxicity

		***Exacum lawii* ethanolic extract (Dose)**				
Haematological parameters	**units**	**Control**	**1000 mg/kg**	**2000 mg/kg**	**4000 mg/kg**	**Satellite**
WBC	$x10^9/l$	8.05±0.54	8.23±0.48	7.95±0.45	8.43±0.33	8.8±0.29
RBC	$x10^{12}/l$	5.68±0.23	6.15±0.17	5.84±0.19	6.9±6.8	7.21±0.39
Platelet	$x10^9/l$	750.34±8.9	753.34±7.87	752.5±5.7	760.16±7.7	769.5±9.24
HB	gm/l	160.67±3.8	161.84±5.03	162.5±6.16	164.34±2.7	166.83±2.8
Hematocrit	%	36.76±0.36	37.43±0.31	36.6±0.29	37.11±0.70	39.87±1.57

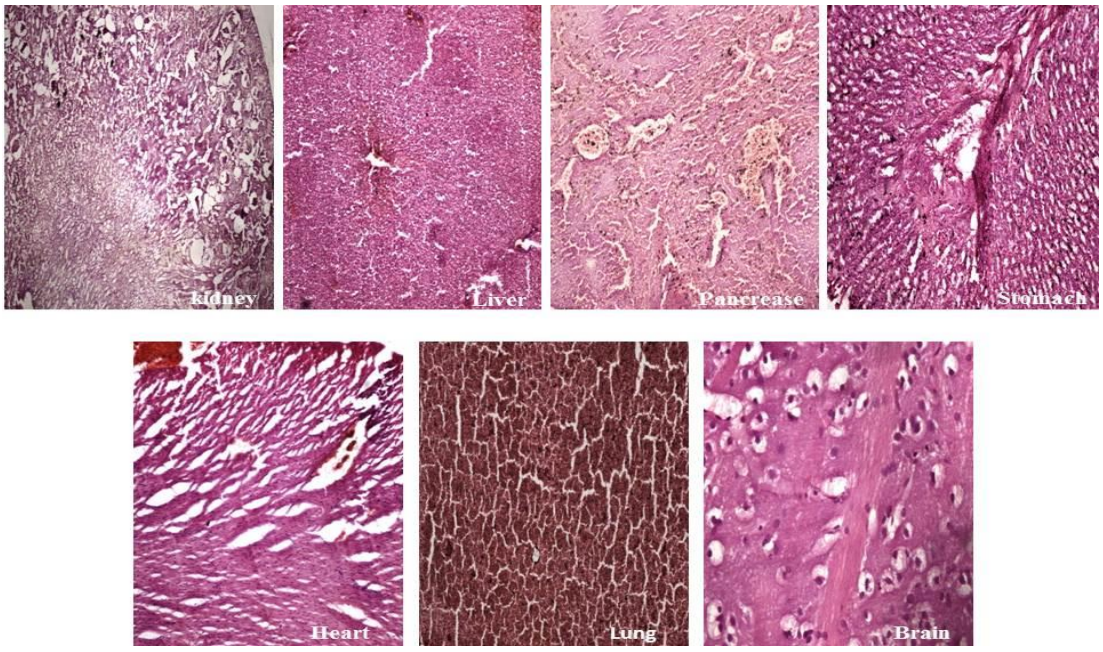
Values are mean ± SEM, (n=10). One way ANOVA followed by Newman–Keuls Multiple Comparison test, *P value <0.05, **P value <0.01, ***P value <0.001.



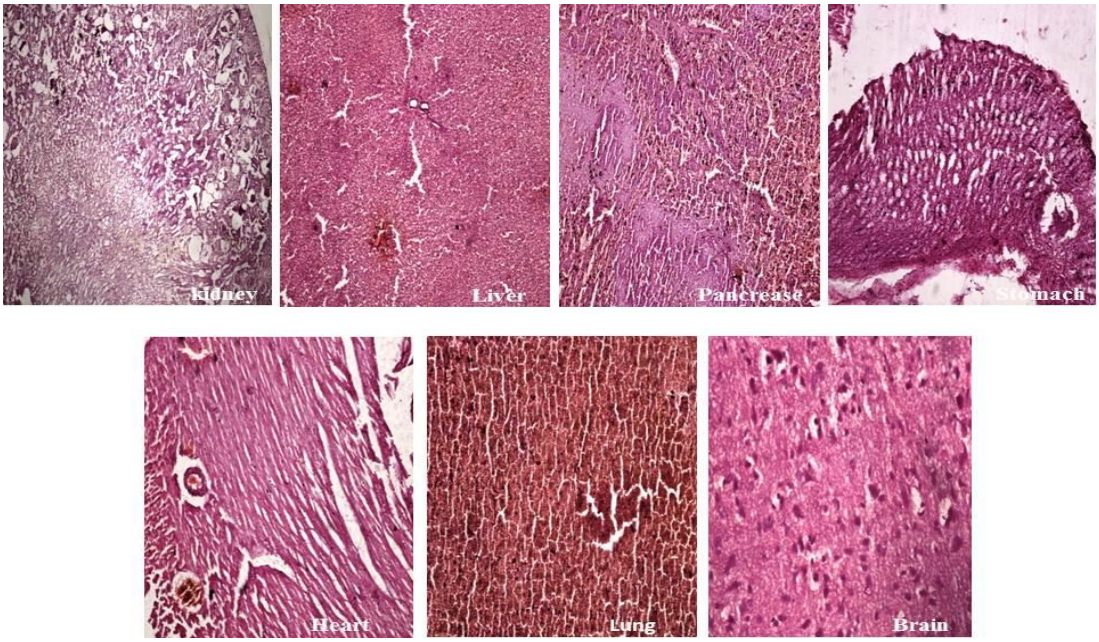
Group I: Histopathological images of Control group



Group II: Histopathological images of group treated with 1000 mg/kg of ELE



Group III: Histopathological images of group treated with 2000 mg/kg of ELE

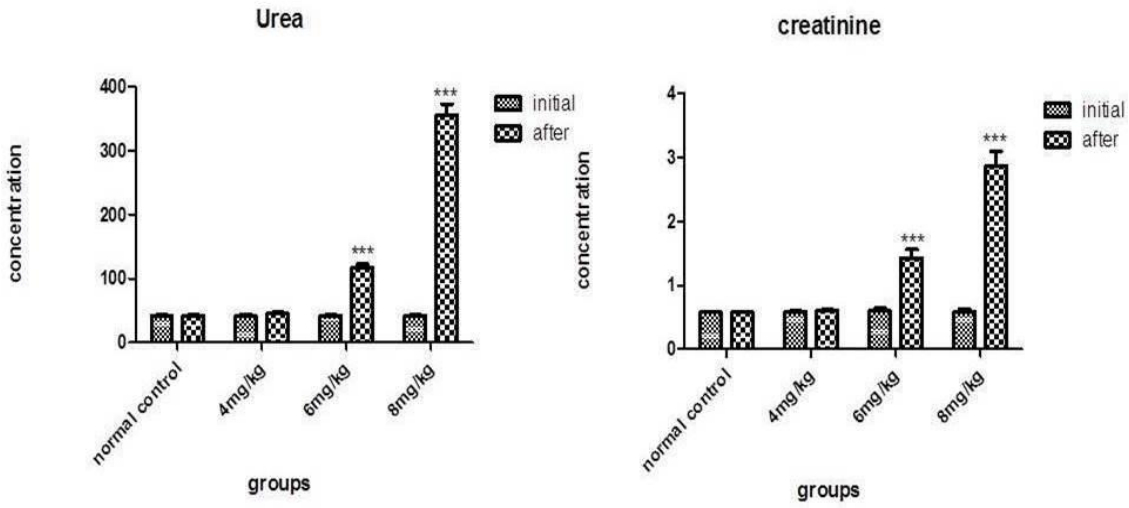


Group IV: Histopathological images of group treated with 4000 mg/kg of ELE

Figure 24: Histopathology of various vital organs during oral toxicity study

1.2. Dose standardisation of cisplatin

Cisplatin (4 mg/kg, i.p.) had not shown any significant elevation ($P>0.05$) in urea and creatinine level after 72 hours of cisplatin administration. Cisplatin at dose of 6 mg/kg, i.p. and 8 mg/kg, i.p. caused significant elevation ($P<0.05$) in urea and creatinine concentration. Administration of cisplatin at the dose of 8 mg/kg, i.p., causes mortality (one half) in a group of tested rats. Therefore, nephroprotective activity of ELE had been evaluated in rats treated with 6 mg/kg, i.p of cisplatin (Figure 25)**.**



Values are mean ± SEM, (n=6). One way ANOVA followed by Newman-Keuls Multiple Comparison test, *P value <0.05, **P value <0.01, ***P value <0.001, comparing with control group.

Figure 25: Effect of different doses of cisplatin on blood urea and creatinine concentration

1.3. Determination of biochemical parameters in serum

Cisplatin induces kidney injury markers like serum urea and creatinine and also raises some biochemical markers. Pre and post treatment with ELE (400 mg/kg, p.o.) and Swertiamerin (20 mg/kg) for seven days was found to lower these markers significantly (P<0.05) and balanced the biochemical markers up to normal level as compared with control (Table 19).

Table 19: Effect of different doses of *Exacum lawii* extract and swertiamerin on serum biochemical parameters in rats treated with cisplatin (6mg/kg, i.p.)

Parameters	Unit	Control	Toxic control	ELE100	ELE200	ELE400	S20
Urea	mg/dl	46.86±0.09	71.92±2.17^{aef}	70.22±2.3^{adef}	62.35±2.12^{abef}	49.05±2.5^{bcd}	49.82±2.5^{bcd}
Creatinine	mg/dl	0.39±0.004	0.59±0.04^{a}	0.58±0.027^{a}	0.52±0.032^{a}	0.40±0.05^{bc}	0.43±0.05^{b}
SGOT	IU/l	137.13±0.67	170.06±3.4^{aef}	160.15±7.92^{ae}	155.5±6.70	139.67±5.27^{bcd}	142.83±5.56^{b}
SGPT	IU/l	65±4.97	88.05±3.42^{aef}	82.75±3.20^{a}	80.7±3.74	68.88±4.36^{b}	69.85±4.32^{b}
TP	gm/dl	7.42±0.38	5.95±0.28^{ae}	6.0±0.41^{ea}	6.11±0.37^{ae}	7.6±0.37^{bcd}	7.098±0.33^{d}
TB	mg/dl	1.23±0.22	4.40±0.58^{af}	4.45±0.77^{af}	3.02±0.45	1.38±0.26^{bc}	3.02±0.32
DB	mg/dl	0.42±0.04	1.08±0.02^{af}	0.99±0.07^{af}	0.75±0.07	0.39±0.16^{bc}	0.46±0.17^{bc}
ALP	IU/L	68.37±3.15	258.34±13.8^{aef}	253.34±8.61^{aef}	242±10.22^{aef}	72.67±5.7^{bcd}	77.83±4.67^{bcd}

Values expressed as means ± SEM (n = 6) of 6 animal in each group. a: $p < 0.05$, comparing with normal control group; b: $p < 0.05$, comparing with toxic control group; c: comparing with treatment group (ELE 100 mg/kg, p.o); d: $p < 0.05$, comparing with treatment group (ELE 200 mg/kg, p.o); e: $p < 0.05$, comparing with treatment group (ELE 400 mg/kg, p.o); f: $p < 0.05$, comparing with treatment group (S20 mg/kg, p.o). (One way analysis of variance (ANOVA) followed by Newman–Keuls Multiple Comparision test).

1.4. Determination of antioxidant parameters in renal tissue homogenate

The activity of SOD, Catalase and GSH content had decreased while MDA level in cisplatin (6 mg/kg, i.p.) treated rat was found be elevated might be due to increasing ROS in renal tissues. ELE (400 mg/kg, p.o.) and swertiamerin (20 mg/kg) treatment significantly inverted the changes in antioxidant enzymes in dose-dependent manner ($p<0.05$) (Table 20).

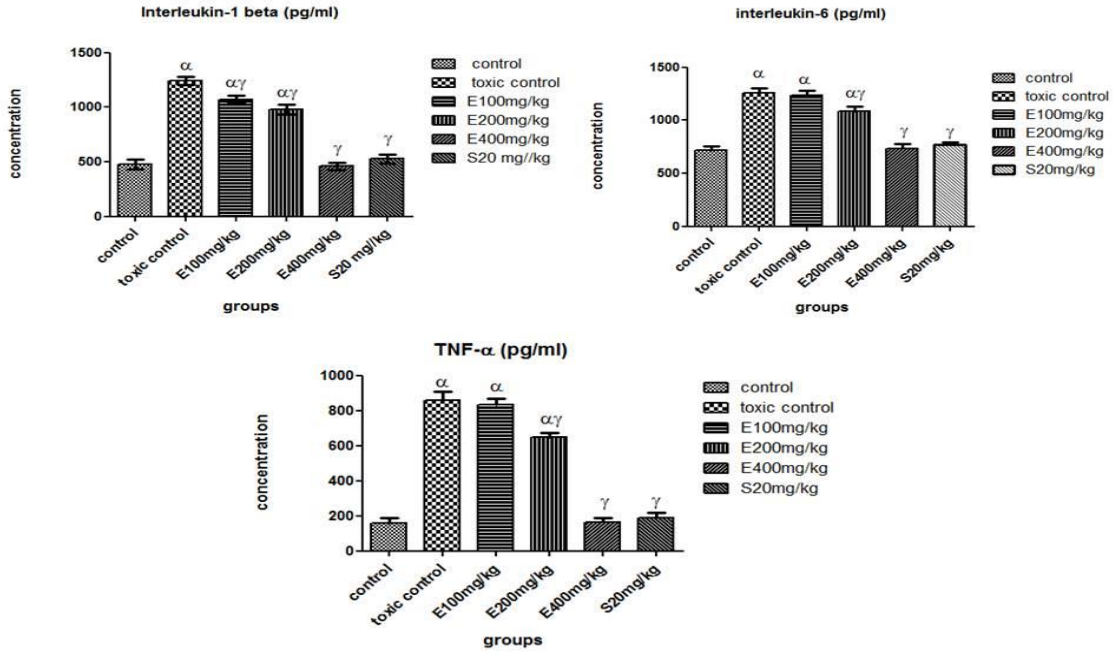
Table 20: Effect of different doses of *Exacum lawii* extract and swertiamerin on renal tissue antioxidant enzyme levels in rats treated with cisplatin (6 mg/kg, i.p.)

Parameters	Unit	Control	Toxic control	ELE100	ELE200	ELE400	S20
CAT	µmol H2O2 consumed/min /mg of protein	28.46±0.83	12.03 ± 1.0^{aefd}	13.49 ± 0.93^{aef}	16.21 ± 0.99^{abef}	27.0 ± 0.87^{bcd}	25.8 ± 1.01^{bcd}
LPO	nmol MDA/g of tissue	24.58±2.3	61.93 ± 7.91^{aef}	57.73 ± 7.6^{aef}	44.67±7.9	26.32 ± 8.12^{bc}	28.50 ± 4.83^{bc}
GSH	µg glutathione per mg protein	264.2±27.64	123.2 ± 36.8^{aef}	128.8 ± 49.05^{aef}	195.6±18.26	262.0 ± 17.7^{bc}	269.2 ± 20.3^{bc}
SOD	Units/ mg protein	36.38±0.33	14.65 ± 3.43^{aef}	18.07 ± 1.86^{aef}	22.29 ± 1.50^{aef}	37.15 ± 0.55^{bcd}	31.50 ± 3.53^{bcd}

Values expressed as means ± SEM (n = 6) of 6 animal in each group. a: $p < 0.05$, comparing with normal control group; b: $p < 0.05$, comparing with toxic control group; c: comparing with treatment group (ELE 100 mg/kg, p.o); d: $p < 0.05$, comparing with treatment group (ELE 200 mg/kg, p.o); e: $p < 0.05$, comparing with treatment group (ELE 400 mg/kg, p.o); f: $p < 0.05$, comparing with treatment group (S20 mg/kg, p.o). (One way analysis of variance (ANOVA) followed by Newman–Keuls Multiple Comparision test).

1.5. Determination of proinflammatory in renal tissue homogenate

The proinflammatory cytokines in rat kidney tissues were investigated in-vitro enzyme-linked immunosorbent assay by measuring cytokine levels of TNF α, IL-6 and IL-1β on homogenised renal tissues using specific antibodies coated 96-well plates. The level of TNF-α, IL-6 and IL-1β in serum was found to be elevated in cisplatin-treated rats. However, ELE (400 mg/kg, p.o.) and swertiamerin (20 mg/kg) treatment had reduced the expression of these proinflammatory molecules in the kidney tissues (Figure 26).

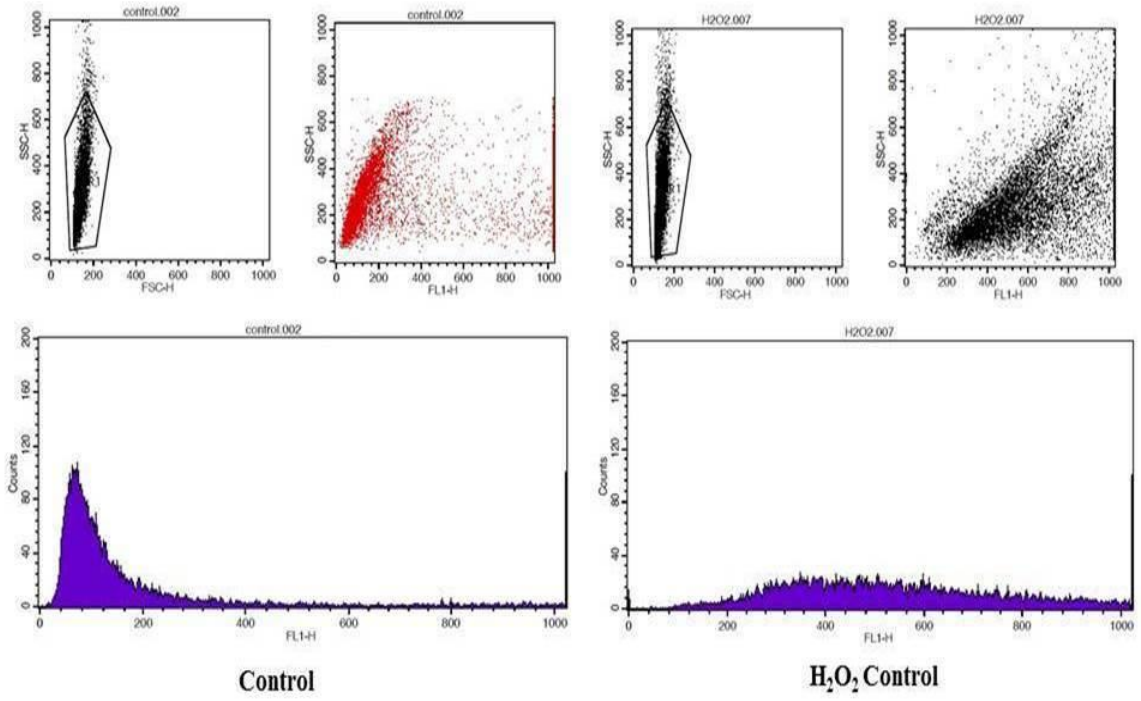


Values expressed as means ± SEM (n = 6) of 6 rats in each group. α: $p < 0.05$, comparing with normal control group; γ: $p < 0.05$, comparing with toxic control group.

Figure 26: Effect of ELE (100mg/kg, p.o., 200mg/kg, p.o., 400mg/kg, p.o.) and swertiamerin (20mg/kg, p.o.) treatment on proinflammatory cytokines (IL-1β, and TNF-α) level in HEK-293 treated with cisplatin

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

Flow cytometry analysis of intracellular ROS production by kidney cells of experimental rats after exposure to cisplatin. H_2O_2 treated kidney cells were taken as external control. Data for Geometrical mean (Geo Mean ± SD) values of fluorescence intensities for each group from two independent experiments is represented in the Bar graph [Figure 4]. Overlay histogram showed the fluorescent intensities of ELE (400 mg/kg, p.o.), Swertiamerin (20 mg/kg, p.o.), cisplatin (6 mg/kg, i.p.) treated, control and unstained cells (Figure 27-28).



a

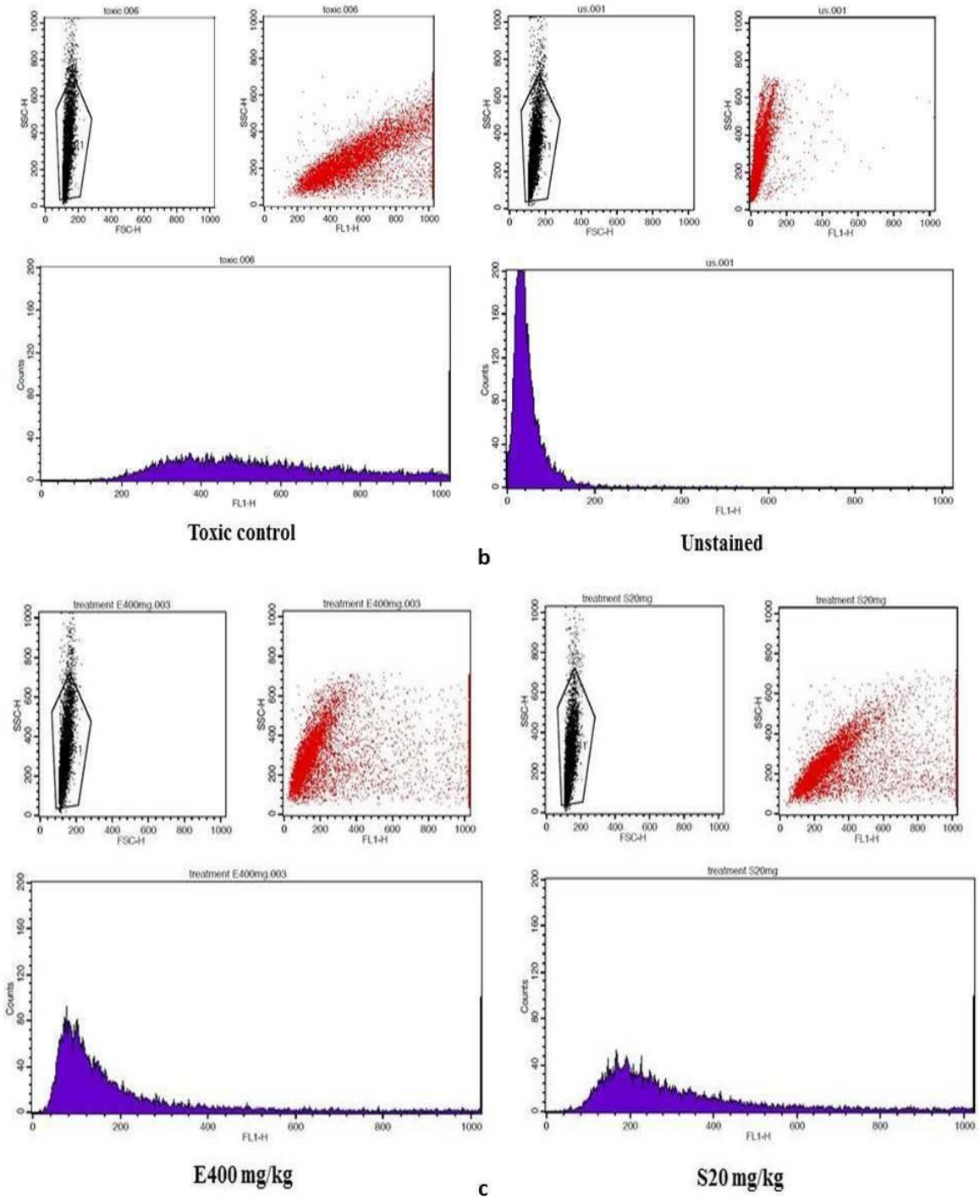
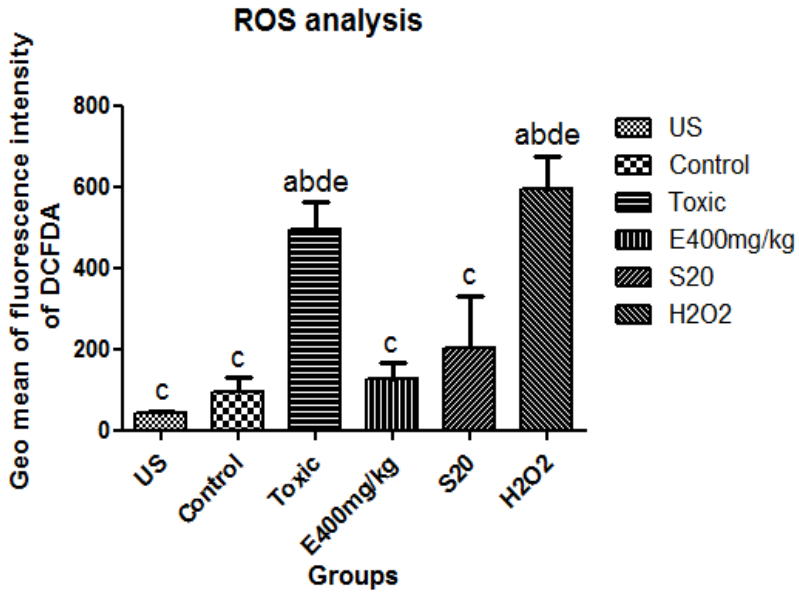


Figure 27: Histogram showing Flow cytometric analysis of ROS production in kidney cells of respective treatment group. Along with dot plot of flow cytometric analysis represents live cells a) control group and external control (H_2O_2) group. b) Toxic control (cisplatin treatment) and unstained group. c) Treatment of *Exacum lawii* extract (E400mg/kg) and Swertiamerin (S20mg/kg). x-axis: Fluorescent intensity, y-axis: cell count.



Values expressed as means ± SEM (n = 3). a: $p < 0.05$, comparing with unstained cells, b: $p< 0.05$, comparing with control group; c: $p < 0.05$, comparing with toxic group; d: $p < 0.05$, comparing with E400 mg/kg., p.o. group; e: $p < 0.05$, comparing with S20 mg/kg., p.o. group

Figure 28: Graph showing Geometrical mean (GeoMean) values of the fluorescence intensities of respective groups.

1.7. Qualitative DNA fragmentation assay

To further define the mechanism involved in cisplatin-induced nephrotoxicity the DNA fragmentation assay against marker 1kb ladder was performed. Cisplatin administration led to DNA fragmentation. Cisplatin-induced DNA breakage at multiple positions across chromosomal DNA may leads to apoptosis. Rats treated with ELE (400 mg/kg, p.o.) and Swertiamerin (20 mg/kg, p.o.) showed very less or no DNA fragmentation (Figure 29).

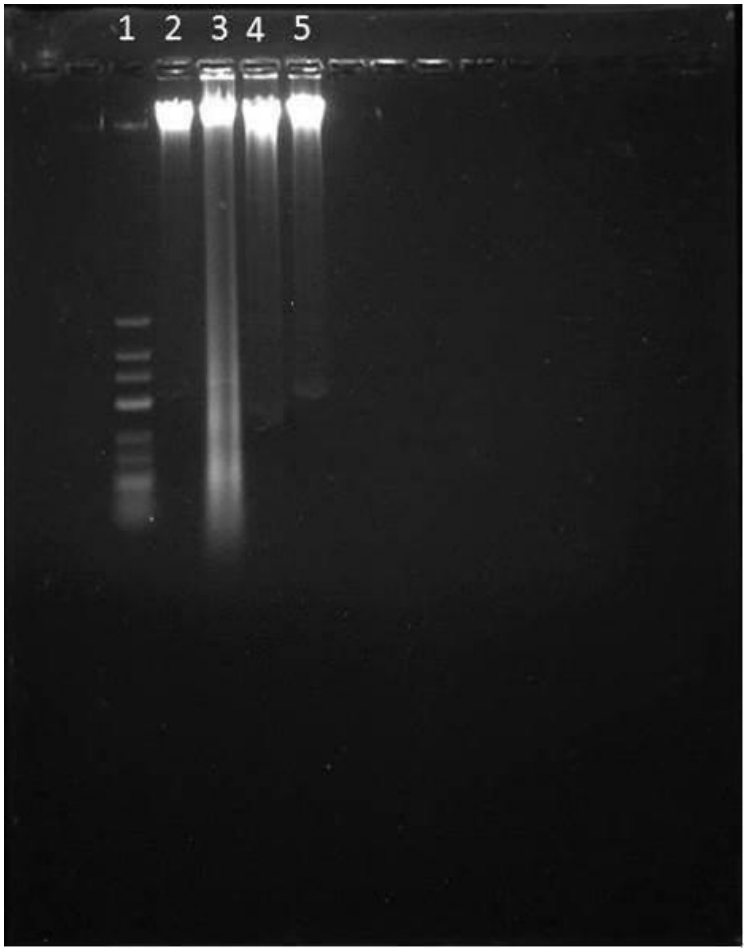


Figure 29: DNA fragmentation of renal cells exposed to cisplatin. Each lane reflecting the presence of DNA fragments was viewed on an ethidium bromide-stained gel. Lane 1: Marker, Lane 2: control group, Lane 3: Cisplatin treated group, Lane 4: ELE 400 mg/kg treated, Lane 5: Swertiamerin 20 mg/kg treated.

1.8. Histopathological study

Cisplatin treatment showed prominent alteration and loss in normal architecture of renal tissues. The renal histology of toxic group was found with distorted histology with atrophied glomerulus, collecting tubules with necrosis. Rats treated with ELE (100 mg/kg, p.o. and 200 mg/kg, p.o.) showed no prominent protective effect on cisplatin-induced nephrotoxicity. Treatment with ELE (400 mg/kg, p.o.) and swertiamerin (20 mg/kg, p.o.) justified the protective action by minimizing

renal vein congestion, tubular necrosis, inflammation and vacuolization in renal histology and cortex was observed with normal histology of convoluted tubules and medulla with collecting tubule and pars recta (Figure 30).

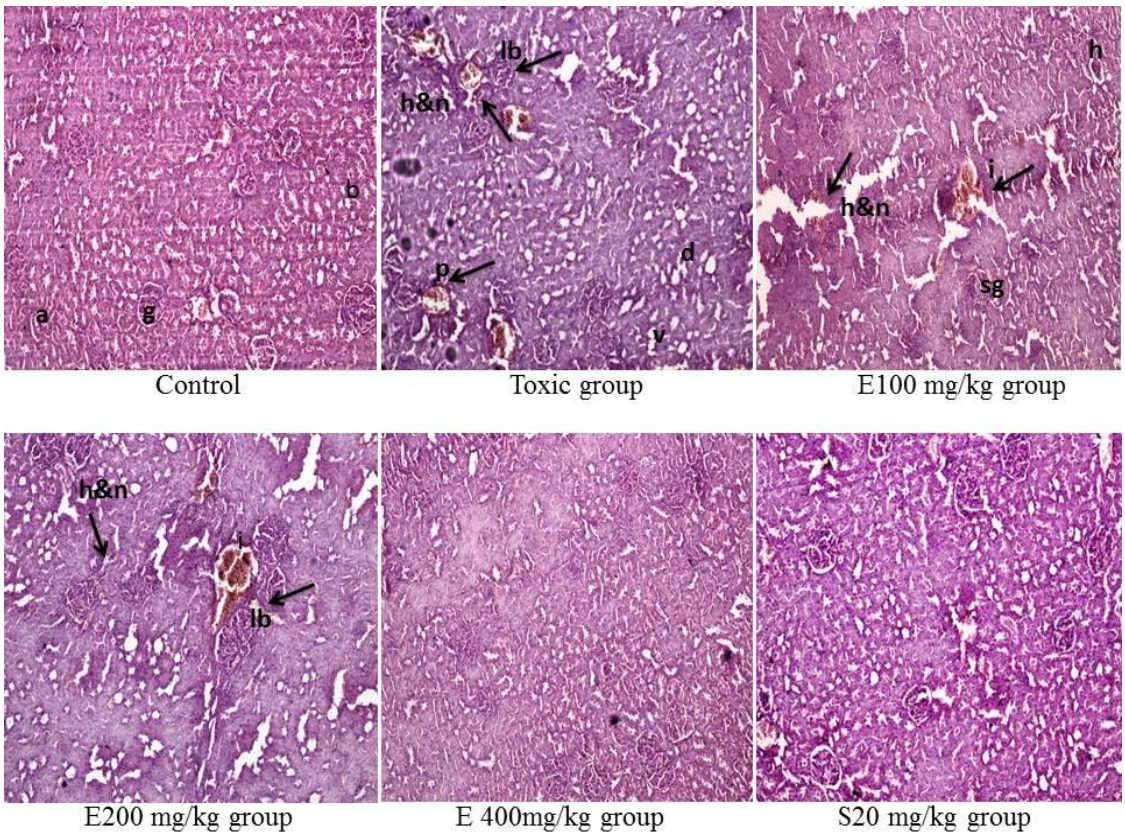


Figure 30: Photomicrographs of Periodic acid-Schiff reagents stained kidney tissue sections showing the protective effect of ELE on cisplatin induced renal injury in experimental rats. a: collecting tubule; b: convoluted tubule; g: glomerulus; p: collagen deposition around parietal layer of the Bowman`s capsule; d: Distal convoluted tubules showed variable degrees of dilatation; v: cytoplasmic vacuolization lb: lobulated and shrunken glomeruli; h&n: hemolysis and necrosis i: inflammation; h: hyaline cast.

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

The Cell proliferation assay was performed to evaluate the cytotoxic effects of Cisplatin, Swertiamerin and ELE against HEK-293 cell line and selecting the dose for further study. Relative cell viability for HEK-293 cell lines was measured at seven different concentrations of extract and compound. The obtained growth curve for cisplatin showed that the inhibition of cell growth was dose dependent (Fig. 1). The inhibitory concentration 50% (IC_{50}) value was found to be 2.261µg/ml. Swertiamerin and ELE did not inhibit the cell proliferation, therefore had no IC_{50} value (Figure 32).

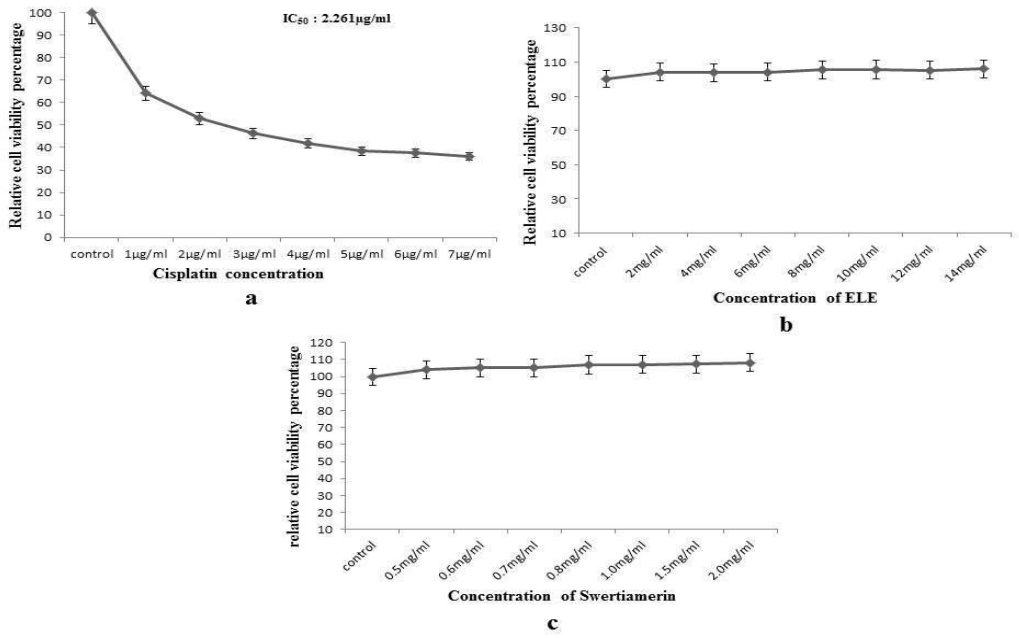


Figure 31: Effect of cisplatin, ELE and swertiamerin on cell viability. HEK 293 cells were treated with various concentrations of cisplatin for 48 hours.

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

We validated the downregulation of iNOS in cisplatin administered HEK-293 by ELE and swertiamerin. Blot was compared to the respective untreated cells (Figure 32).

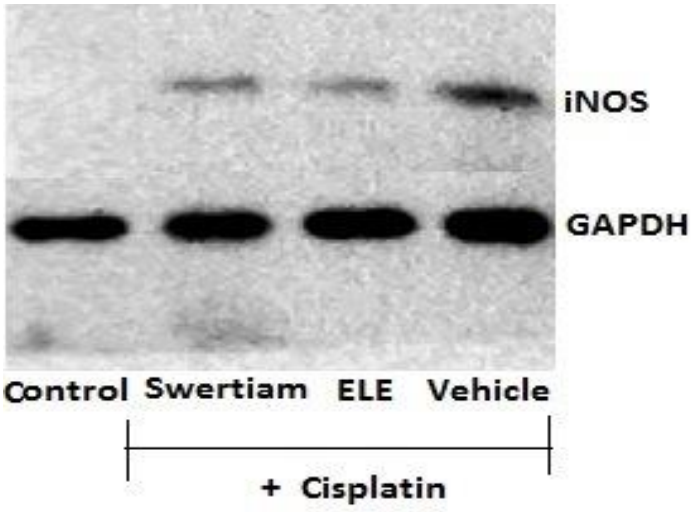
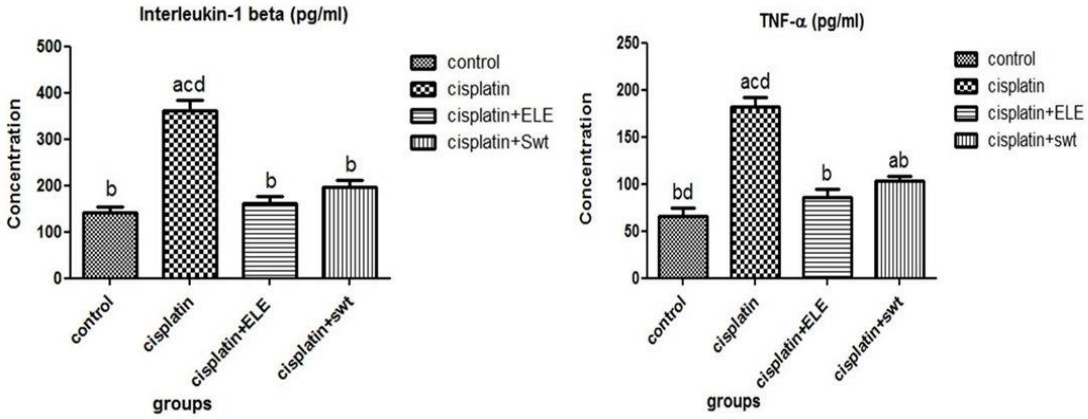


Figure 32: Cisplatin induced marked increases in protein iNOS, ELE and Swertiamerin attenuate the cisplatin-induced renal overexpression of iNOS. The blots were stripped and reprobed for GAPDH protein as a loading control.

2.3. Estimation of Pro-inflammatory cytokine

Cisplatin (1µg/ml) administration in HEK-293 results in the upregulation of a level of proinflammatory cytokines (IL-1β and TNF-α). Treatment with ELE (2 mg/ml) and swertiamerin (0.5 mg/ml) demonstrated that the level of proinflammatory cytokines in HEK-293 was lowered to normal level (Figure 33).



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).

Figure 33: Proinflammatory cytokines TNF-α and IL-β in HEK-293 treated HEK-293 cells with cisplatin (1 mg/ml), ELE (2 mg/ml) and swertiamerin (0.5 mg/ml)

2.4. 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. H_2O_2 treated kidney cells were taken as external control. Data for Geometrical mean values of fluorescence intensities for each group. H_2O_2 treated kidney cells were taken as external control (Figure 34).

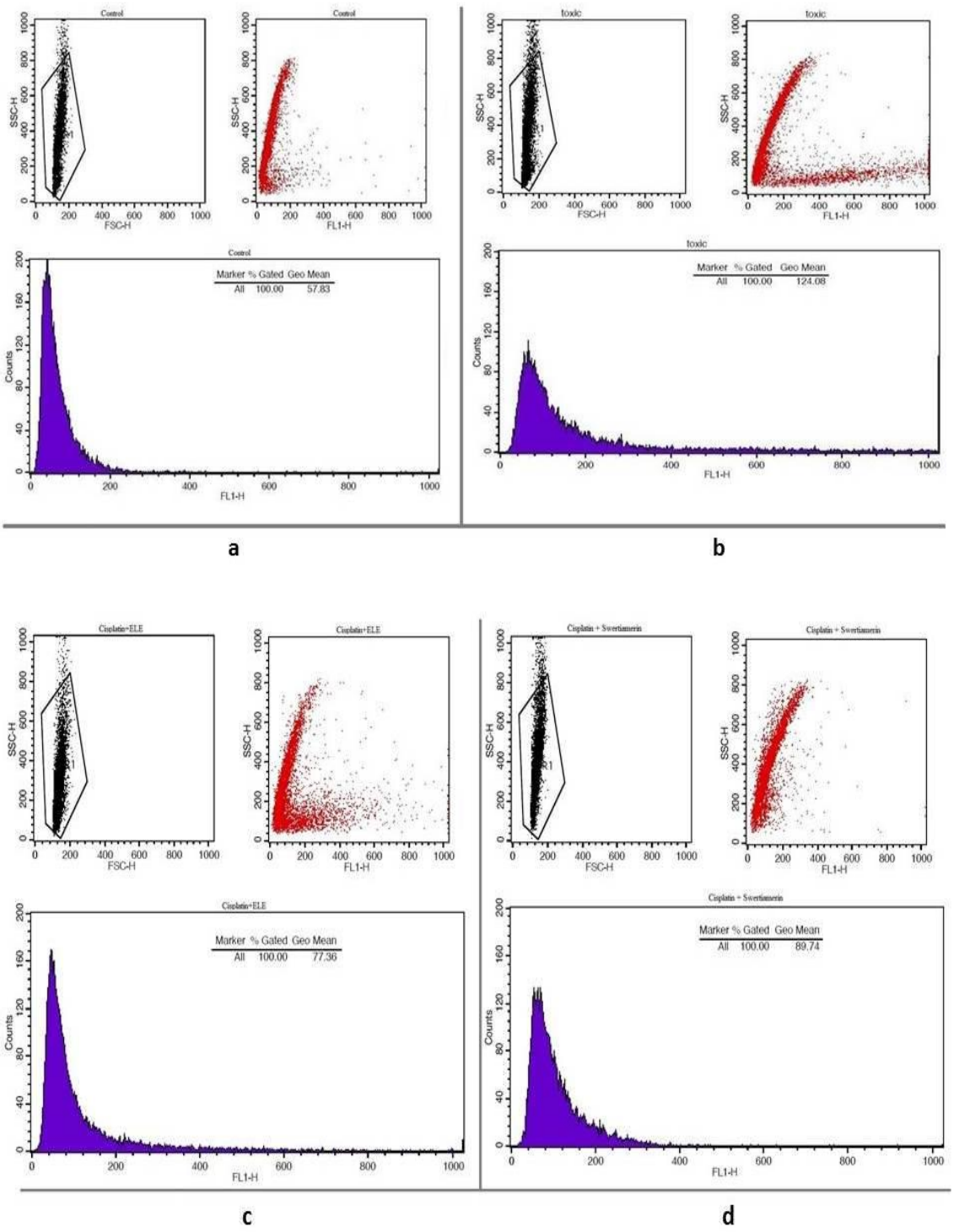


Figure 34: Flow cytometry analysis of intracellular ROS production by HEK-293 cells, a) untreated cells b) cells after exposure to cisplatin c) cells after exposure to cisplatin along with ELE treatment and d) cells after exposure to cisplatin along with swertiamerin treatment.

2.5. DNA Fragmentation assay

Fragments were observed in DNA content of cisplatin treated cells against 1 kb ladder, ELE (2mg/ml) and swertiamerin (0.5 mg/ml) prevent the DNA damage by reducing the damage in DNA content of HEK-293 cells treated with cisplatin when compared with control (Figure 35).

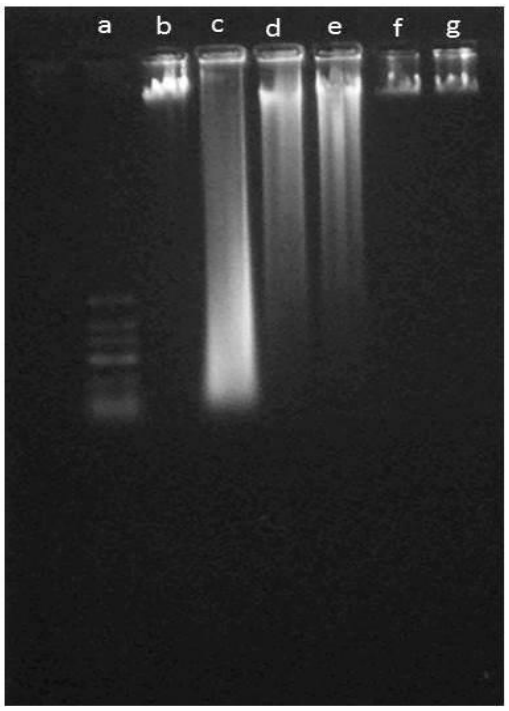


Figure 35: DNA fragmentation of HEK-293 cells exposed to cisplatin. Each lane reflecting the presence of DNA fragments was viewed on an ethidium bromide-stained gel. Lane a: Marker, Lane b: DNA of untreated cells, Lane c: DNA of cisplatin treated cells, Lane d: DNA of cisplatin + ELE (2mg/ml) treated, Lane e: DNA of cisplatin + Swertiamerin (0.5mg/ml) treated, Lane f: DNA of ELE (2mg/ml) alone, Lane g: DNA of Swertiamerin (0.5mg/ml) alone.

2.6. Cell cycle analysis

FACS analysis determines the distribution of cells in various stages of the cell cycle. The result showed that, after cisplatin administration the cells enter into cell cycle and the percentage of G2/M phase in cisplatin treated HEK-293 was found to increase upto 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 in (Figure 36).

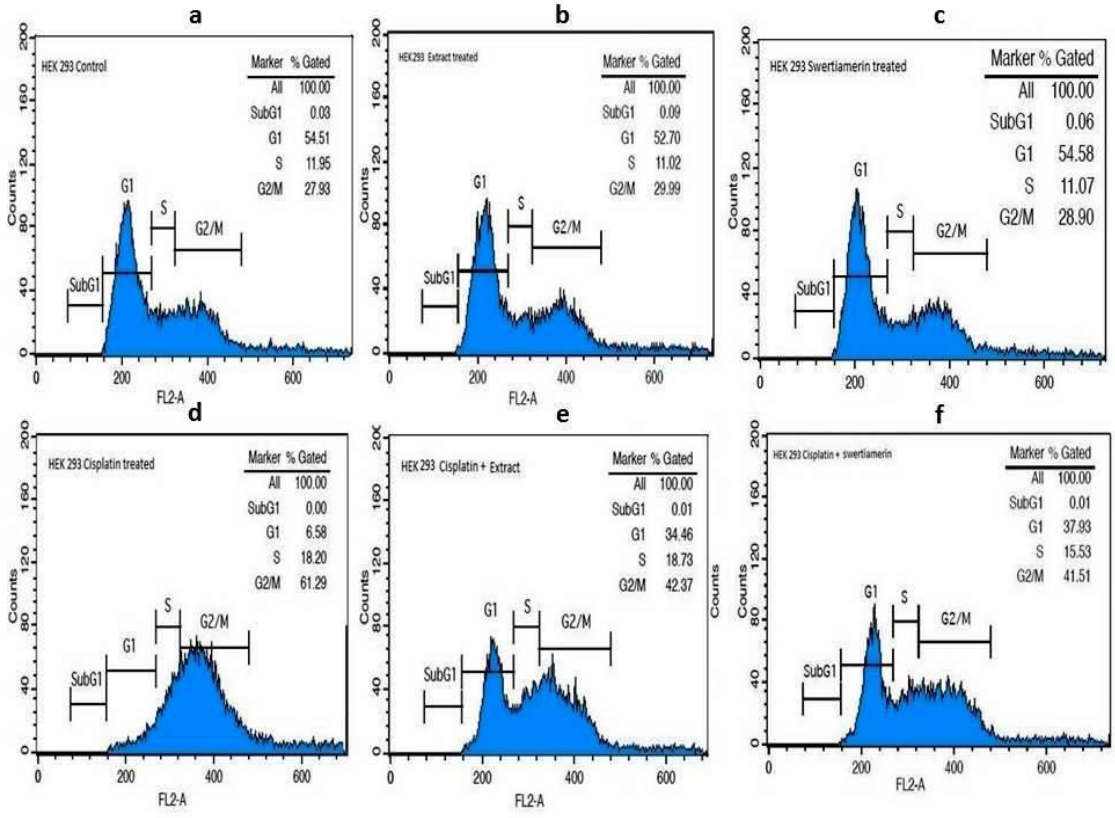


Figure 36: Flowcytometric analysis showing distribution of HEK-293 cell line in various phases of cell cycle, a: untreated cells, b: ELE treated alone, c:

swertiamerin treated alone, d: Cisplatin treated, e: cisplatin + ELE (2mg/ml) treated, f: cisplatin + Swertiamerin (0.5mg/ml) treated.

2.7. Morphological analysis

As shown in figure 37, untreated HEK-293 cells were adherent, flattened thin elongated with up-to 80% confluency. Cisplatin administered cells were floating and less adherents, with more apoptic bodies, cytoplasmic vacuoles, shrinking cells, shape becomes more spherical, formation of blebs on the surface. Treatment with ELE and swertiamerin helps the cells to conserve their shape and showed less abnormalities (Figure 37).

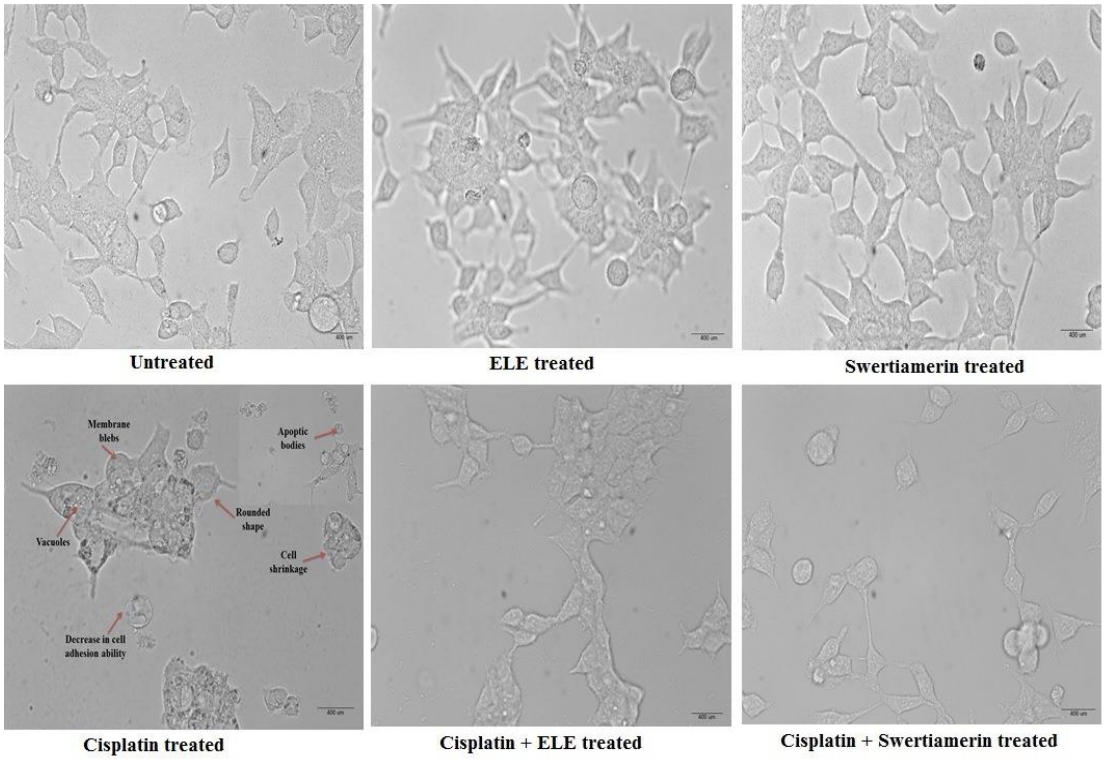
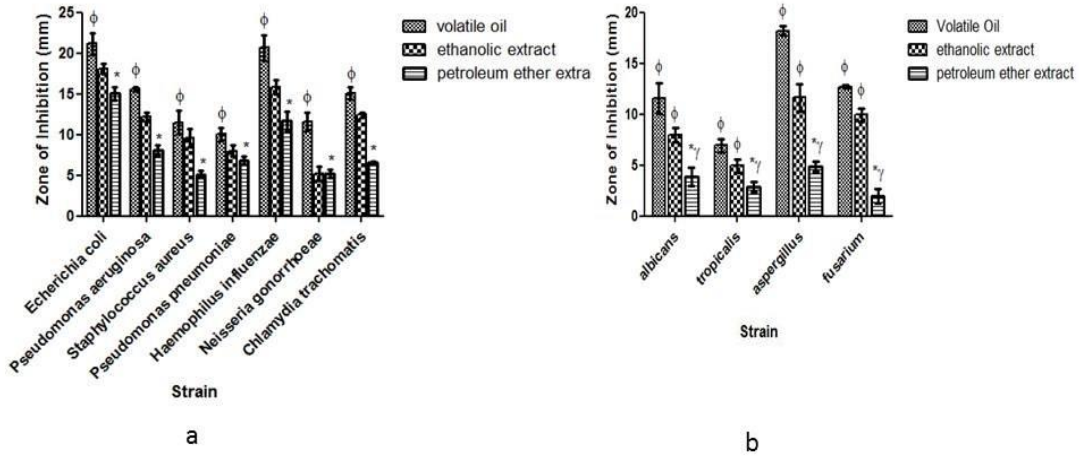


Figure 37: Morphology of HEK-293 cell line in untreated condition, treatment with ELE alone and swertiamerin alone showed adherent, flattened and elongated morphology. Cisplatin exposure causes cells less adherent, produce apoptic bodies, formation of blebs, shrinkage of cell membrane. Cisplatin+ELE and

Cisplatin+ swertiamerin recover the toxic symptoms and conserve the normal morphology.

3. Antimicrobial Susceptibility Test

Activities of different extracts against the test organisms were expressed as zone of inhibition (in mm). The zone of Inhibition for bacterial strain causing ocular infection for volatile oil ranged between 10.05±0.78 to 21.15±1.34, for ethanolic extract 5.20± 0.85 to 18.05 ± 0.64 and for petroleum ether extracts 5.10±0.43. The zone of Inhibition for pathogenic fungal strain causing ocular infection ranges between 6.95±0.63 to 18.20 ±0.42 (volatile oil), 4.95±0.63 to 11.65±1.34 (ethanolic extract) and 0 to 8.00±0.7 (petroleum ether extract) (Figure 38).



Values are expressed as means±SD. *P<0.05, comparing Zone of Inhibition for volatile oil; Φ P<0.05, comparing Zone of Inhibition for petroleum ether extract of *Exacum lawii*; γ P<0.05, comparing Zone of Inhibition for ethanolic extract of *Exacum lawii*. One-way ANOVA followed by Newman–Keuls multiple comparison tests.

Figure 38: Zone of Inhibition for volatile oil and extracts of *Exacum lawii* against a) pathogenic bacterial strains causing ocular infection, b) pathogenic fungal strains causing ocular infection.

3.1 Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC)

MBC and MFC for volatile oil of *Exacum lawii* against various bacterial and fungal strains causing ocular infection ranged between 3.125 mg/ml to 12.25 mg/ml and 3.125 mg/ml to 12.5 respectively. MBC and MFC ranged between 6.25 mg/ml to 25 mg/ml and 12.5 mg/ml to 25 mg/ml for ethanolic extract respectively and 25 mg/ml to 50 mg/ml to 25 mg/ml to 50 mg/ml for petroleum ether extract respectively, against various bacterial and fungal strain causing ocular infection. MBC and MFC for standard (Himalaya Optha-care Eye Drop) against various bacterial and fungal strains causing ocular infection ranged from 1.56 mg/ml to 6.25 mg/ml (Table 21-22).

Table 21: MIC and MBC (mg/ml) values of *Exacum lawii* extracts and volatile oil against bacterial strain causing ocular infection

Bacterial strain	**Gram +ve/-ve**	**MIC/MBC (mg/ml)**			
		Volatile oil	**Ethanolic extract**	**Petroleum ether extract**	**Standard**
Echerichia coli	-ve	3.125/6.25	6.25/12.25	25/50	1.56/3.125
Pseudomonas aeruginosa	- ve	3.125/3.125	6.25/6.25	25/25	3.125/3.125
Staphylococcus aureus	+ ve	3.125/3.125	6.25/6.25	25/25	1.56/3.125
Pseudomonas pneumoniae	+ ve	6.25/12.25	12.25/25	25/25	3.125/6.25
Haemophilus influenzae	- ve	3.125/3.125	12.25/12.25	25/25	1.56/3.125
Neisseria gonorrhoeae	- ve	6.25/6.25	12.25/25	25/50	3.125/6.25
Chlamydia trachomatis	- ve	6.25/12.5	25/25	25/50	3.125/6.25

Table 22: MIC and MFC (mg/ml) values of *Exacum lawii* extracts and volatile oil against fungal strain causing ocular infection

	MIC/MFC (mg/ml)			
Fungal strains	**Volatile oil**	**Petroleum ether extract**	**Ethanolic extract**	**Standard**
Candida albicans	3.125/3.125	25/25	12.5/12.5	1.56/3.125
Candida tropicalis	12.5/12.5	25/25	12.5/12.5	6.25/12.5
Aspergillus keratitis	6.25/6.25	50/50	25/25	3.125/6.25
Fusarium dimerum	6.25/6.25	25/50	12.5/12.5	1.56/6.25