Pharmacogn J. 2018; 10(6)Suppl: s71-s76
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
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Original Article

In vitro Cytotoxicity Study on U87 Cells using Root Extracts of Plumbago Species and GC-MS Study

Sandhya Panicker[1,*], Veluthat Kolangara Haridasan[2]

ABSTRACT

Plumbago is a genus of medicinal plants that are used in recent years to induce significant levels of apoptosis in various cancer cells. **Purpose:** The test substances that are obtained from Plumbago species are studied for their cytotoxicity against U87 cell line in a dose dependent manner and were first subjected to GC-MS study to know the bioactive constituents present in them. **Methods:** GC-MS was done using ethanol extracts of the roots of both the species. *In vitro* cytotoxicity of the roots of two species of Plumbago –*P. zeylanica* (sample I) and *P. auriculata* (sample II) were tested against U87 cell line. Test samples were taken at concentrations ranging from 400µg/ml to 3.12µg/ml to determine the percentage growth inhibition of both the test substances on U87 cell line. **Results:** GC-MS analysis on root extracts of *P. zeylanica* showed the presence of 27 phytochemical constituents and *P. auriculata* 16 in number .The test substances, Sample I and Sample II exhibited a cytotoxic CTC_{50} value of 88.07±4.4 and 23.11±0.9 respectively. **Conclusion:** *P. zeylanica* is more effective than *P. auriculata i*n terms of its cytotoxicity as well in the number of useful bioactive compounds.
Key words: Cytotoxicity, U87 cell line, GC-MS, Bioactive constituents.

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History

- Submission Date: 21-05-2018;
- Review completed: 15-07-2018;
- Accepted Date: 01-08-2018

DOI : 10.5530/pj.2018.6s.14

Article Available online
http://www.phcogj.com/v10/i6s

INTRODUCTION

Plumbagin, an active constituent found in various parts of the plant Plumbago was studied extensively against various cancer cell lines such as P388 lymphocytic leukemia[1] non-small cell lung cancer cell lines (A549, H262 and H460)[2] and apoptosis pathway in breast cancer and lung cancer as well. It was also found to inactivate the oncogenic transcription factor Forkhead Box M1 (FOXM1) signalling pathway in glioma cell.[3] Plumbagin induce apoptosis in human pancreatic cancer cells primarily through the mitochondria-related pathway which indicates that plumbagin can be potentially developed as a novel therapeutic agent against pancreatic cancer also.[4]

Gas Chromatography Mass Spectroscopy is a very compatible technique and most commonly used for the identification and quantification purpose. The unknown organic compounds in a complex mixture can be identified by matching the spectra with reference spectra.[5]

In vitro measurement of toxicity is purely a cellular pathway which can be measured by changes in cell survival or its metabolism.[6] To test cell proliferation and cell survival; a simple calorimetric assay was developed by Mosmann[7] which was further modified for the measurement of chemosensitivity and cytotoxicity on human malignant cell lines. This assay is called MTT assay. The MTT assay involves the ability of viable cells to convert a soluble tetrazolium salt, (3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) (MTT) into an insoluble formazan precipitate by the mitochondrial succinate dehydrogenase enzyme which are active in living cells.[8]

MATERIALS AND METHODS

GC –MS STUDY

Preparation of the extract: The pulverized root powder was successively extracted with ethanol. The extracts were then concentrated under reduced pressure in a rotary evaporator.

Extract: 1µl each of the ethanol extracts of the roots of *P.zeylanica* and *P.auriculata* was employed for GC –MS analysis.[5]

Instruments and chromatographic conditions: The analysis was performed on a Shimadzu GC-MS (Model Number: QP2010S) equipped with column consisting of Rxi-5Sil MS (30 meter length, 0.25 mm ID and 0.25 µm thickness). The instrument was operated at 70 eV in electron impact mode. Helium (99.995%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of split ratio 50:0. Column Oven Temperature was at 80.0°C, Injection Temperature- 260.00°C, Pressure- 65.2 kPa, Total Flow- 54.0 mL/min, Linear Velocity: 36.8 cm/sec, Purge Flow: 3.0 mL/min. The total GC time was 45 minand the MS time was 40.00 min. The relative amounts of individual components of the total composition were expressed as percentage peak area relative to total peak area.

Cite this article: Panicker S, Haridasan VK. *In vitro* Cytotoxicity Study on U87 Cells Using Root Extracts of Plumbago Species and GC-MS Study. Pharmacog J. 2018;10(6)Suppl:s71-s76.

Identification of the compounds: Interpretation on mass spectrum of GC-MS was done using GCMS Software- GCMS Solutions and the libraries used were NIST 11 and WILEY 8.

In vitro Cytotoxicity Studies

Material used: The roots of two species of Plumbago –*P.zeylanica* (sample I) and *P. auriculata* (sample II) were shade dried- pulverized and extracted using ethanol. The extracts were allowed to vacuum evaporate and the residues deposited on the walls of the chamber was collected and used for the study. The *in vitro* cytotoxicity was performed for "Sample I and Sample II" on Human Glioblastoma cells to find toxic concentration of test substances.

Test solution: For cytotoxicity studies, 100µl each of the test drug was separately suspended and volume was made up with Ham's F12 supplemented with 2% inactivated Fetal Bovine Serum to obtain a stock solution of 10% v/v concentration. This was sterilized by 0.22µ syringe filtration. Cytotoxicity studies were carried out using two fold serial dilutions from this.

Cell line and Culture medium: U87 (Human Glioblastoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in Ham's F12 supplemented with 10% inactivated Fetal Bovine Serum, Penicillin (100 IU/ml), streptomycin (100µg/ml) and Amphotericin B (5µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% Trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well micro titre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Cytotoxicity Studies: The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using Ham's F12 containing 10% FBS. To each well of the 96 well micro titre plates, 0.1 ml of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer washed once with medium and 100µl of different test concentrations of test drugs were added on to the partial monolayer in micro titre plates. The plates were then incubated at 37°C for 72 h in 5% CO_2 atmosphere, and microscopic examination (Figure 2) was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100µl of propane was added and the plates were gently shaken to solubilise the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 540 nm.[9] The percentage growth inhibition was calculated using the standard formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values was generated from the dose-response curves (Figure 2) for each cell line.[10]

$$\% \text{ Growth Inhibition} = \left(\frac{\text{Mean OD of individual test sample}}{\text{Mean OD of control}} \times 100 \right)$$

RESULTS AND DISCUSSION

GC-MS study: *P. zeylanica* showed the presence of 27 phytochemical (Table 1) constituents where 1, 4-naphthalenedione, 5-hydroxy-2-methyl-(23.75%) was in the highest concentration. In *P. auriculata* (Table 2) 1, 4-naphthalenedione, 5-hydroxy-2-methy-(34.12%) was found in highest concentration followed by lupeol (20.41%). Plumbagin or 5-hydroxy-2-methyl-1,4-napthoquinone is derived naphthoquinone isolated from the roots of the *Plumbago zeylanica* L. (common name- Chitrak). It is anti-atherogenic, cardiotonic, hepatoprotective and neuroprotective agent. Topical application of non-toxic doses (100-500 nmol) of PL to skin elicits dose-dependent inhibition of ultraviolet radiation (UVR)-induced development of squamous cell carcinomas (SCC).[11] Lupeol isolated from *Elaeodendron buchananii* exhibited potent anticancer activity against LEUK-L1210 cells.[12]

***In vitro* Cytotoxicity Studies:** Test substances "Sample I (*P.zeylanica*) and Sample II" (*P. auriculata*) were tested for *in vitro* cytotoxicity studies against Human Glioblastoma (U87) using MTT assay exposing the cells to different concentrations of test substances (Figure 1). The test

Table 1: GC-MS study on the ethanolic extract of *P. auriculata* roots indicating 16 phytochemical constituents.

Peak	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	5.543	53304	0.15	33402	0.59	propane, 1,1-diethoxy-	0.15
2	11.781	216140	0.60	147237	2.58	phenol, 2,4-bis(1,1-dimethylethyl)-	0.60
3	13.151	3961134	10.91	1946249	34.12	1,4-naphthalenedione, 5-hydroxy-2-methyl-	10.91
4	14.137	162117	0.45	71009	1.25	benzaldehyde, 4-ethoxy-	0.45
5	16.796	377057	1.04	192687	3.38	hexadecanoic acid	1.04
6	18.325	916860	2.53	88476	1.55	methyl commate c	2.53
7	18.451	586769	1.62	191587	3.36	1,3,12-nonadecatriene	1.62
8	18.500	763845	2.10	181563	3.18	7-tetradecenal, (z)-	2.10
9	18.712	34925	0.10	19960	0.35	9,12-octadecadienoic acid (z,z)-	0.10
10	19.867	719267	1.98	191823	3.36	longifolenaldehyde	1.98
11	20.082	12326744	33.96	1164217	20.41	lupeol	33.96
12	21.725	873134	2.41	103854	1.82	methyl commate b	2.41
13	22.836	8722260	24.03	803615	14.09	nerolidyl acetate	24.03
14	25.322	1807563	4.98	127714	2.24	stigmast-4-en-3-one	4.98
15	36.058	3379206	9.31	324173	5.68	hexadecanoic acid, 9-octadecenyl ester, (z)-	9.31
16	36.297	1400911	3.86	115965	2.03	2,6,10-trimethyl,14-ethylene-14-pentadecne	3.86

Table 2: GC-MS study on the ethanolic extract of *P. zeylanica* roots indicating 27 phytochemical constituents.

Peak	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	5.545	54879	0.19	33784	0.75	Propane, 1,1-diethoxy-	59.05
2	11.781	177214	0.62	122003	2.70	Phenol, 2,4-bis(1,1-dimethylethyl)-	191.15
3	13.148	2264904	7.94	1073113	23.75	1,4-Naphthalenedione, 5-hydroxy-2-methyl-	188.05
4	14.133	406586	1.43	168324	3.73	Ethanone, 2-ethoxy-1-phenyl-	121.10
5	14.780	46406	0.16	14586	0.32	14,15,16-Trinorlabd-12-ene, 8,13-Epoxy-	95.10
6	15.068	124657	0.44	77103	1.71	(-)-Drimenol	109.10
7	15.217	16297	0.06	9986	0.22	3-Dodecanol	59.05
8	15.333	52053	0.18	33754	0.75	1,3-Cyclopentadiene, 1,2,5,5-tetramethyl-	107.15
9	15.631	1137474	3.99	602867	13.34	2,4-Heptadiene, 2,6-dimethyl-	109.15
10	15.938	441516	1.55	232018	5.14	1-Cycloheptene, 1,4-dimethyl-3-(2-methyl-1-propene-1-yl)-4-vinyl-	107.10
11	16.092	111469	0.39	64266	1.42	Alpha-guaien	119.15
12	16.478	98123	0.34	47289	1.05	(3E)-5-Isopropylidene-2,7-dimethyl-6-oxa-1,3,7,10-UNDECATETRAENE	109.15
13	16.796	316003	1.11	148661	3.29	Hexadecanoic acid	60.05
14	16.980	148984	0.52	76614	1.70	Drimenin	109.15
15	17.123	206640	0.72	136051	3.01	Hexadecanoic acid, ethyl ester	88.10
16	17.659	11015	0.04	11153	0.25	6-Oxa-3,3,5-trimethyl-spiro[5,2]octa-5-ene	219.10
17	18.012	118002	0.41	70332	1.56	Retinol, acetate	119.10
18	18.076	60376	0.21	29933	0.66	5,8-Decadien-2-one, 5,9-dimethyl-, (E)-	96.10
19	18.450	67235	0.24	30608	0.68	(5E)-5-Dodecen-1-ol #	67.05
20	18.503	144072	0.50	53962	1.19	9-Octadecenoic acid (Z)-	55.05
21	18.711	178162	0.62	100360	2.22	ethanol, 2-(9,12-octadecadienyloxy)-, (z,z)-	67.10
22	18.768	167481	0.59	110750	2.45	7-Tetradecenal, (Z)-	55.05
23	20.855	19664	0.07	10458	0.23	1-(2-Hydroxyethoxy)tridecane	57.10
24	34.565	20501964	71.86	1035744	22.92	11-Dodecen-1-ol difluoroacetate	55.05
25	35.106	1101235	3.86	109261	2.42	Z,E-2,13-Octadecadien-1-ol	55.05
26	35.167	342950	1.20	80014	1.77	17-Oxolinoleic acid, methyl ester	91.05
27	39.338	213990	0.75	35182	0.78	22,23-Dibromostigmasterol acetate	105.10

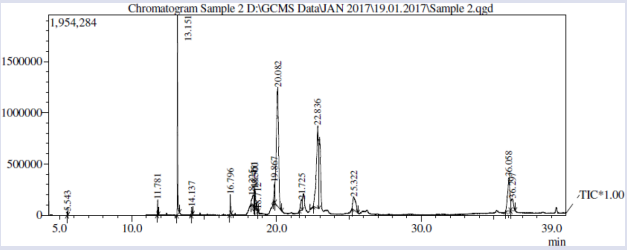


Figure 1: GC-MS Chromatogram of ethanolic extracts of *P. auriculata* roots.

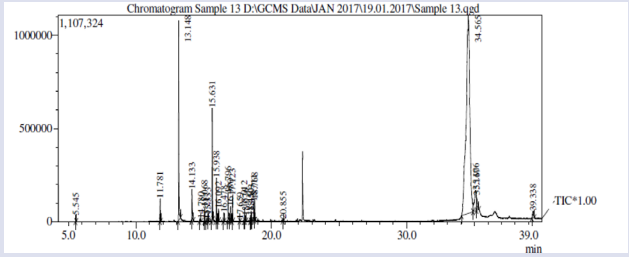


Figure 2: GC-MS Chromatogram of ethanolic extracts of *P. zeylanica* roots.

substances, Sample I and Sample II exhibited a CTC_{50} (concentration of the sample tolerated by 50% of the cultures exposed) value of 88.07 ± 4.4 and 23.11 ± 0.9 respectively (Table 4). The antitumorous activity of the sample was recorded in a dose dependent manner. Drugs that target cancerous cells prevent its growth and division, become cytotoxic in nature targeting nucleic acids and their precursors that are rapidly synthesized during cell division.[13] The side effects may be acute or chronic, self-limited, permanent, mild or potentially life threatening.[14]

CONCLUSION

From the current studies it can be inferred that the ethanolic root extract of *P. zeylanica* contained a larger number of bioactive compounds (Table

Table 3: Some of the bioactive compounds identified using ethanolic extracts of *P. auriculata* and *P. zeylanica* roots.

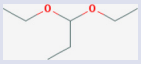
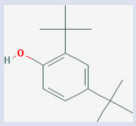
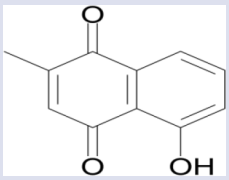
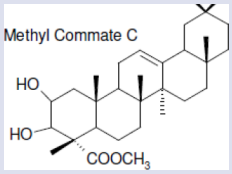
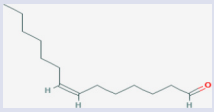
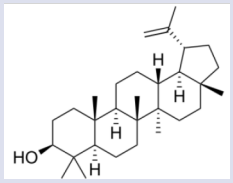
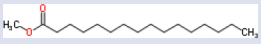
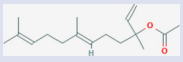
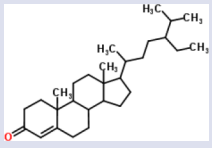
Sl. no	Compound name	Structure	Function
1	Propane, 1,1-diethoxy-		Food additives -Flavouring Agents
2	Phenol, 2,4-bis(1,1-dimethylethyl)-		Solvent for cleaning or degreasing
3	1,4-Naphthalenedione, 5-hydroxy-2-methyl-		antimicrobial antimalarial, anti-inflammatory, anticarcinogenic, cardiotonic, immunosuppressive, antifertility action, neuroprotective, anti-atherosclerosis effects
4	Benzaldehyde, 4-ethoxy-		4-Ethoxybenzaldehyde is present in black tea. 4-Ethoxybenzaldehyde is a flavouring agent.
5	Hexadecanoic acid		Negative feedback on acetyl-CoA carboxylase (ACC) which prevents palmitate generation.
6	Methyl commate c		Antibacterial, anti-inflammatory
7	7-Tetradecenal, (z)-		Agrochemical Category- Attractant
8	9,12-Octadecadienoic acid (z,z)-		Food additives - Flavoring Agent
9	Longifolenaldehyde		anticancer and antibacterial activities
10	Lupeol		antiprotozoal, antimicrobial, anti-inflammatory, antitumor and chemopreventive properties
11	Methyl commate b		Triterpenes glycoside
12	Nerolidyl acetate		Flavour and fragrance agent
13	Stigmast-4-en-3-one		Hypoglycaemic effect

Table 3: Cont'd.

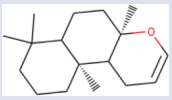
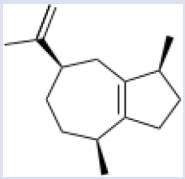
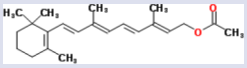
14	14,15,16-trinorlabd-12-ene, 8,13-epoxy-		Used in perfumery
15	3-Dodecanol		Surfactants, lubricating oils, pharmaceuticals, in the formation of monolithic polymers and as a flavour enhancing food additive. In cosmetics, used as an emollient. It is also the precursor to dodecanal, an important fragrance.
16	Alpha-guaien		fragrance and flavouring industries
17	Hexadecanoic acid		soaps, cosmetics, and industrial mold release agents
18	Hexadecanoic acid, ethyl ester		hair- and skin-conditioning agent
19	Retinol acetate		Anti neoplastic and chemo preventive activities
20	9-octadecenoic acid (z)-		Insect pheromone

Table 4: Cytotoxic properties of test drugs against U87 cell line.

Sl. No.	Name of Test compound	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	Sample I	400	70.13±1.1	88.07±4.4
		200	58.87±1.6	
		100	51.77±1.1	
		50	44.92±1.3	
		25	33.10±3.0	
		12.5	18.83±1.21	
		6.25	15.92±1.12	
		3.12	6.15±1.65	
2	Sample II	400	74.39±0.6	23.11±0.9
		200	71.16±0.9	
		100	61.86±0.8	
		50	58.08±0.9	
		25	52.48±1.6	
		12.5	36.80±1.5	
		6.25	32.07±1.2	
		3.12	13.40±1.0	

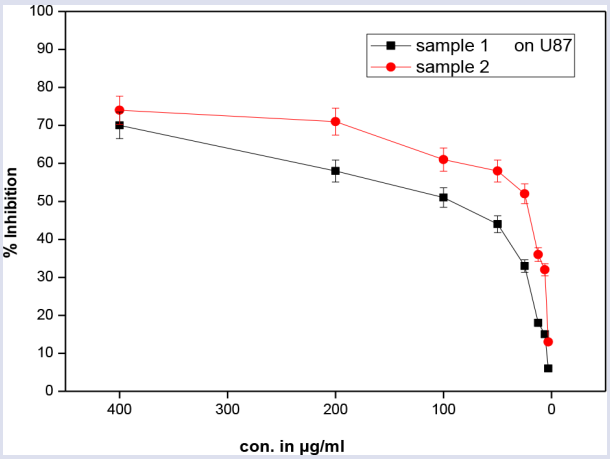


Figure 1: Graph showing cytotoxic effect of test substances on U87 cell line.

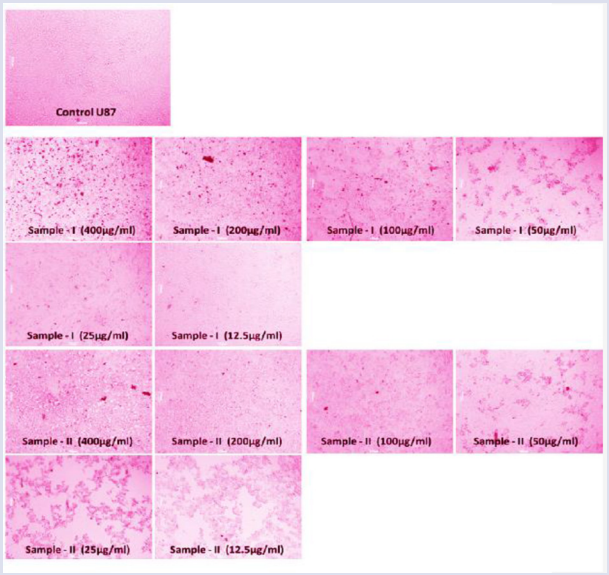


Figure 2: Microscopic observation showing cytotoxicity of Sample I and Sample II on U87 cell line.

3) compared to *P. auriculata* as revealed through the GC-MS studies. It has also been observed that the cytotoxicity is more in *P. zeylanica* on U87 (Human Glioblastoma) cell line than *P. auriculata* using MTT assay. Hence the potential of the roots of *P. zeylanica* can be further exploited for cancer therapy.

ACKNOWLEDGEMENT

The authors wish to thank Department of Botany, Post Graduate and Research Centre, St. Joseph's College -Bangalore for its valuable support in carrying out the studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

GC-MS: Gas Chromatography Mass Spectroscopy; **SCC**: Squamous cell carcinomas; **U87**: Human Glioblastoma cell line; **CTC_{50}**: Concentration of the sample tolerated by 50% of the cultures exposed; **UVR**: Ultraviolet radiation.

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Cite this article: Panicker S, Haridasan VK. *In vitro* Cytotoxicity Study on U87 Cells using Root Extracts of Plumbago Species and GC-MS Study. Pharmacog J. 2018;10(6)Suppl:s71-s76.