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GC-MS ANALYSIS AND "*IN VITRO*" ANTICANCER ASSAY OF METHANOLIC LEAF EXTRACT OF *ASYSTASIA GANGETICA (L.)*. T.ANDERSON AGAINST A549 HUMAN LUNG CANCER CELL LINE

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ABSTRACT

As lung cancer is one of the most notorious cancer worldwide and the mortality rate is expected to increase drastically in developing countries like China and India, the present investigation has been made in order to get a remedy from medicinal plant. In the present study the *"in vitro"* anticancer assay of Methanolic Leaf extract of *Asystasia gangetica* (MLAG) was made against A549 human lung cancer cell lines. The phytoconstituents of the plant has also been identified with the help of GC-MS (Gas Chromatography – Mass Spectrometry) analysis. Numerous anticancer compounds like epithilones, Psi-Psi carotenes, baicalines, veratramines, vitamin-D acetate, colchicines, isocorydines have been identified from the MLAG. The IC_{50} value of MLAG was found to be 122.85µg/ml. It was also found that the cytotoxic value was concentration dependent. As the concentration of MLAG increased the cytotoxic value has found to be increased. The anti-proliferative nature of the plant might be due to the combination of multiple anticancer compounds.

KEYWORDS: *Asystasia gangetica*, GC-MS, MTT Assay, A549 Human Lung Cell Line.

INTRODUCTION

Cancer, known medically as malignant neoplasia, is a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, which may invade nearby parts of the body. The cancer may also spread to more

distant parts of the body through the lymphatic system or blood stream, and is called metasis.[2] Lung cancer, one of the most frequently diagnosed cancers in the world, is characterized with relatively high morbidity and mortality.[14, 25, 27, 12] In developing countries like China and India, the incidence of lung cancer is expected to increase drastically in the next few years. During the past five decades, the incidence of lung adenocarcinoma, a type of non-small cell lung carcinoma started to rise relatively when compared to other types of lung cancer.[5, 3] Over expression of epidermal growth factor receptor, a member of tyrosine kinase receptor has been identified as therapeutic target of several human carcinomas, including non-small cell lung cancer. Recently tyrosine kinase inhibitors have been approved for treatment of lung cancer, but the success rates to these drugs are very low.[10] Chemotherapy is recognized to be the main therapeutic way to delay tumor growth. However, the overall survival remains poor.[17] Therefore, there is an urgent need to identify effective drugs for the treatment of lung cancer. Medicinal plants play a major role in health care system and are exclusive source of life saving drugs for majority of world's population. Anti-cancer activity of medicinal plants is mainly due to presence of phenolic compounds, flavanoids and phenolic diterpenes. Natural products have long been a rich source of cure for cancer. Some of them used in treatment of cancer include taxol, etoposide, topotecan, irinotecan, vincristine, vinblastine, colchicines and ellipticine.[26, 16] *Asystasia gangetica* belonging to the family Acanthaceae commonly known as Chinese violet. It is comprising about 70 species distributed in tropical and subtropical old world regions.[15] *A.gangetica* found to contain numerous important phytochemicals like phenols, alkaloids, flavonoids, glycosides, coumarins, tannins, steroids, terpenoids and saponins. It has also been found to contain four important flavonoids, luteolin, quercetin, kaemferol and isorhamnetin.[6] *A.gangetica* is mainly used for mild hypoglycaemia.[7, 22] It has also been claimed to have anti-asthmatic, antihelminthic, anti-diabetic, anti-oxidant and anti-cancer properties.[1, 7, 13, 19, 28] In Africa, an infusion of the plant is used to ease pain during child birth and the sap is applied to sores, wounds and piles. Powdered root are considered analgesic and used in stomach-ache and snake bites.

MATERIALS AND METHODS

Collection of Plant Materials

Asystasia gangetica(L.) T. Anderson, collected from local area of Thanthondrimalai, Karur, Tamilnadu, India, was used for the present study. The plant was identified and authenticated with Botanical Survey of India (BSI) Southern Circle Coimbatore, Tamilnadu, India.

Preparation of the Extract

The root of the plant was shade dried and powdered. The powder (250g) was subjected to a single soxhlet extraction using methanol (1 liter) for 24hrs. The extract was then concentrated to dryness under reduced pressure and controlled temperature to yield dark brown semisolids (Yield 6.85% and 5.24%) which was preserved in refrigerated condition till further use.

GC-MS Analysis

The methanolic extract of the plant root powder was injected by hypodermic syringe into the inlet port of GC. The full scan MS of the compounds were measured from m /z 80 – 750. MS data were acquired in the negative ionization mode. The results can be expressed in terms of retention time (Rt), which is the time required for elution of sample or RV – the volume of carrier gas required to elute a component from the column. These parameters are nearly always expressed in terms relative to a standard compound (RRv or RRt) which may added to the sample extract or which could take the form of the solvent used for dissolving the sample. GC provides both quantitative and qualitative data on plant substances, since measurements of the area under the peaks shown on the GC trace are directly related to the concentrations of different components in the original mixture.

***In vitro* cytotoxicity- MTT Assay**

MTT-Assay-Chemicals and reagents

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

Cell Lines and Culture Medium

A549 cell cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Medium was supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an 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 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Determination of Cell Viability by MTT Assays

The cleavage of the soluble yellow tetrazolium salt MTT (3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide) into blue coloured formazan by the mitochondrial enzyme succinate dehydrogenase was used for assaying cell survival and proliferation. This assay is extensive used for measuring cell survival and proliferation. There is a direct proportionality between the formazan produced and the number of viable cells. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% FBS and were used for the determination of cell viability by MTT assays as described by Francis and Rita (1986) respectively. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

RESULTS AND DISCUSSION

The mathanolic leaf extract of *Asystasia gangetica* (MLAG) showed many novel phytochemicals like desacetylallocolchicine, rhopalotine, quinazoline, phthalimidomonan, N-Ethyl-deoxy-veratramine, casbene, epothylane A-1, 4-methyl-2 (trichloromethyl) oxazole, rhodoxanthin, cephalotaxine, phenanthroline, baicaline, vitamin E acetate, Psi Psi-carotene, rhodopin, morpholine, etc. GC-MS Chromatogram-1 and Table-1 presented some of the important phytocompounds present in MLAG. Table-2 depicts the medicinal properties of some phytoconstituents present in MLAG.

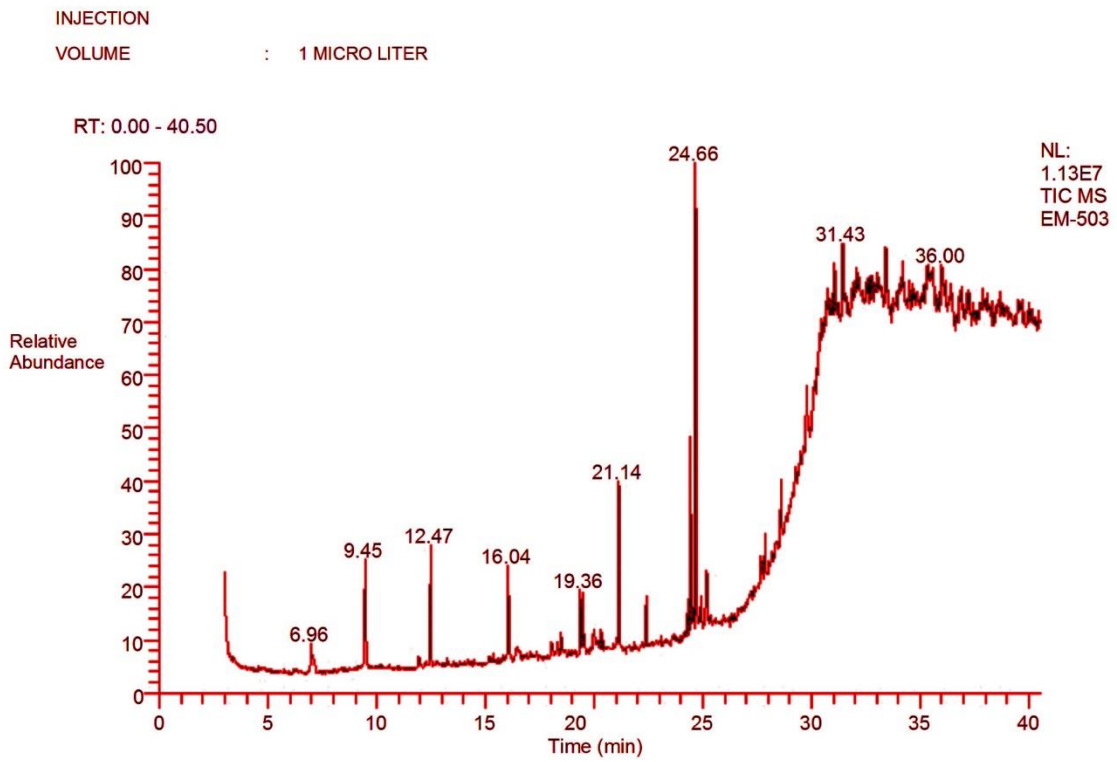


Fig-1.GC-MS Chromatogram *in vivo* Leaf of *A. gangetica.*

Epithilones are a new class of cancer drugs. They prevent cancer cells from dividing by interfering with tubulin, but in early trials epithilones have better efficacy and milder adverse effects than taxanes.[17] Veratramine is a type of hypotensive steroidal alkaloids. It is isolated from the rhizome of *Veratrum dahauricum.*[11] Isocorydine which has also been identified from MLAG is an important aporphine alkaloid. It shows various important pharmacological actions including anti-proliferative, anti-plasmodial, antirrhythmatic, etc.[20] Vitamin E-acetate is tocopheryl acetate and is an excellent moisturizer responsible for hair growth and reduces skin burns and is often used in dermatological products[9]

Colchicine, an alkaloid effectively functions as a mitotic poison or spindle poison.[4] Baicaline a kind of flavonoid prevent hepatocyte apoptosis.[23] Chloestane, a steroid precursor and basic for many organic metabolites has also been isolated from MLAG. Spherodenon otherwise known as Psi, psi carotene is one of the very potent antioxidant carotene also identified from the MLAG. They able to efficiently quench single oxygen because of the presence of several conjugated double bonds and to scavenge free radicals.[24]

***In vitro* Anticancer Assay**

The methanolic leaf extract of *A.gangetica* showed potent anti-proliferative property against A549 human lung cancer cell lines *in vitro* MTT assay. The MLAG of different concentration, viz., 62.5, 125, 250 and 500µg/ml were tested for cytotoxic activity. It was observed that 62.5 µg/ml MLAG showed only minimal cytotoxicity (21.18%) whereas 125µg/ml MLAG showed 50.43% of cytotoxicity. Likewise, 250 and 500µg/ml MLAG showed 69.83% and 91.26% of cytotoxicity respectively. The results obtained were presented in Figure-2 and Table-3. From the result observed it was found that the cytotoxicity of MLAG was found to be dose dependent. The median inhibitory concentration (CTC_{50}) of MLAG was found to be 122.85µg/ml. The dose dependent CTC_{50} value has also been observed in many plants against carcinoma cell lines. Similar results have also been observed in *Cadaba fructicose*,[21] *Paris polyphylla*[8] and *Parthenium hysterophorus.*[18]

Table- 1 Phytoconstituents identified from the methanolic leaf extract of *A. gangetica* through GC-MS analysis.

S.No	R/T	Name of the Compound	Molecular Formula	MW	Peak Area %
1	7.01	Desacetylallocolchicine	C20H23NO5	357	1.57
2	9.47	1,3-Bis(4-chlorobenzyl)-5,6-dihydrobenzo[f]quinazoline	C26H20Cl2N2	430	3.10
3	9.47	bis(trimethylsilyl)-lorazepam	C21H26Cl2	464	3.10
4	9.47	12-Phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline	C26H23NO5	429	3.10
5	16.04	1à,25-Dihydroxy-1á-methylvitamin D3	C28H46O3	430	2.38
6	16.04	Fumaric acid, butyl 2,4-dichloronaphth-1-yl ester)	C18H16Cl2O4	366	2.38
7	18.50	N-Ethyl-desoxy-veratramine	C29H43N	405	1.24
8	20.00	Epothylone A-1	C25H37NO6S	479	1.35
9	21.14	Hexadecanoic acid, methyl ester (CAS)	C17H34O2	270	4.29
10	24.66	á,.Psi.-Carotene, 3',4'-didehydro-1',2'-dihydro-1',2'-dihydroxy-,(2'R)-	C40H56O2	568	22.19
11	25.18	Baicaline	C20H21NO5	355	2.24
12	28.60	1'"-Trimethylsilyl-3-bromo[1-[4-(2-phenyl-1,4-dihexylphenyl)phenyl]]benzene	C39H49BrSi	624	1.25
13	32.10	Molybdenum, tricarbonyltris(trimethylPhosphite-p)-	C12H27MoO12P3	554	3.98
14	33.05	Vitamin E acetate	C31H52O3	472	4.41
15	34.20	Rhodopin	C40H58O	554	3.38
16	35.51	1,3-Bis(3,4,5-trichlorophenyl)triazene	C12H5Cl6N3	401	3.95
17	35.51	4-{2,6-Dibromo-4-[(tert-	C14H19Br2N3O	403	3.95

		butyl)azo]phenyl}morpholine			
18	36.40	Echinenone	C40H54O	550	2.42
19	37.20	Rhodoxanthin	C40H50O2	562	1.53
20	38.63	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	C27H42O4	430	2.09

Table: 2 Pharmacological properties of some of phytocomponents identified in the methanolic root extract of *A. gangetica* by GC-MS.

S.No	R/T	Name of the Compound	Compound Nature	Activity
1	7.01	Desacetylallocolchicine	Alkaloid	Anti-inflammatory, Anticancer, Reduced heart attack
2	9.47	1,3-Bis(4-chlorobenzyl)-5,6-dihydrobenzo[f]quinazoline	Heterocyclic compound	Antimalarial, Anticancer
3	9.47	bis(trimethylsilyl)-lorazepam		Anxiolytic, Chemotherapy drug
4	9.47	12-Phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline	Alkaloids	Anti-hypertension, Antifungal,
5	16.04	1à,25-Dihydroxy-1á-methylvitamin D3	Steroids	Anticancer, Heart disease
6	16.04	Fumaric acid, butyl 2,4-dichloronaphth-1-yl ester)	Crystalline compound	Food additives, Anti-oxidant
7	18.50	N-Ethyl-desoxy-veratramine	Alkaloid	Antimalarial, Anticancer
8	20.00	Epothylone A-1	Organic compound	Anticancer
9	21.14	Hexadecanoic acid, methyl ester (CAS)	Palmitic acid ester	Antioxidant,Hypocholesterolemic Nematicide, Pesticide, Flavor, Lubricant, Antiandrogenic,
10	24.66	á,.Psi.-Carotene, 3',4'-didehydro-1',2'-dihydro-1',2'-dihydroxy-,(2'R)-	Carotenoids	Anticancer, Antioxidant
11	25.18	Baicaline	Flavonoid	Anti-inflammatory, Anticancer
12	28.60	1'"-Trimethylsilyl-3-bromo[1-[4-(2-phenyl-1,4-dihexylphenyl)phenyl]]benzene	Organic chemical compound	Pesticides, Drugs, Dyes
13	32.10	Molybdenum, tricarbonyltris (trimethylPhosphite-p)-	Alkaloid	Fungicide, Fertilizer
14	33.05	Vitamin E acetate	Lipidsoluble compound	Antioxidant
15	34.20	Rhodopin	Carotenoid	Antioxidant
16	35.51	1,3-Bis(3,4,5-trichlorophenyl)triazene	Organic compound	Anticancer Activity
17	35.51	4-{2,6-Dibromo-4-[(tert-butyl)azo]phenyl}morpholine	Organic compound	Anticancer, Antibiotic, Analgesic
18	36.40	Echinenone	Carotenoid	Antioxidant
19	37.20	Rhodoxanthin	Organic compound	Food additives, coloring
20	38.63	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	Fatty acid	Antibacterial, Anti-inflammatory, Anticancer, Fungicide

Table: 3 Cytotoxic Activity of methanolic Leaf Extract of *A. gangetica* on A549 Human Lung Cell Line.

Concentration in µg/ml	% CTC_{50} Cytotoxicity (µg/ml)	CTC_{50}
500	91.26	122.85 µg/ml
250	69.83	
125	50.43	
62.5	21.18	

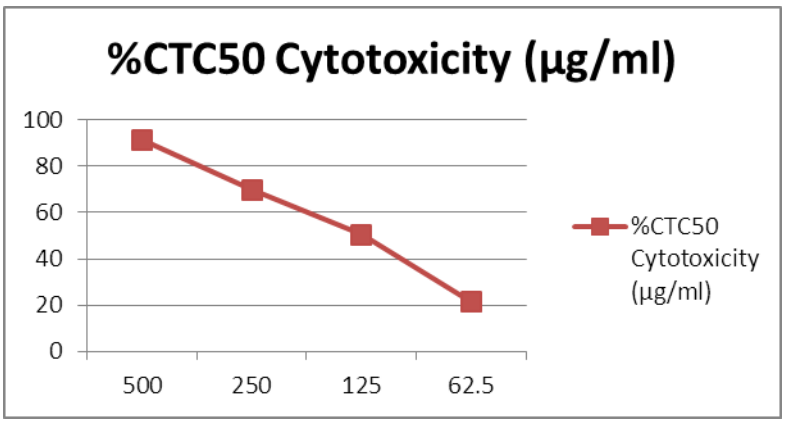
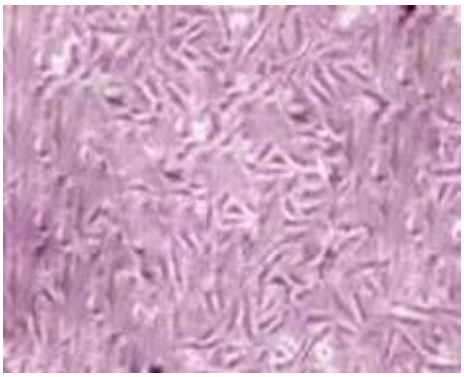
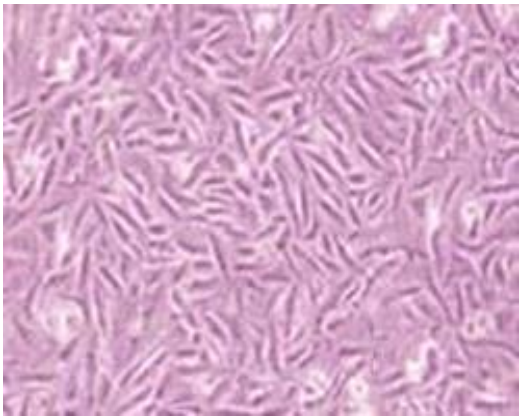


Figure: 2 (A549 Human Lung Cancer).

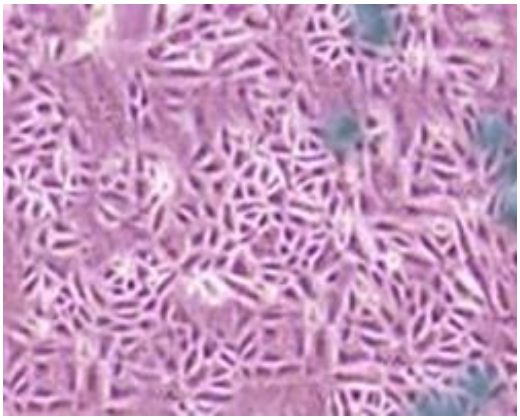
A 549 (Human Lung Cancer cell line)



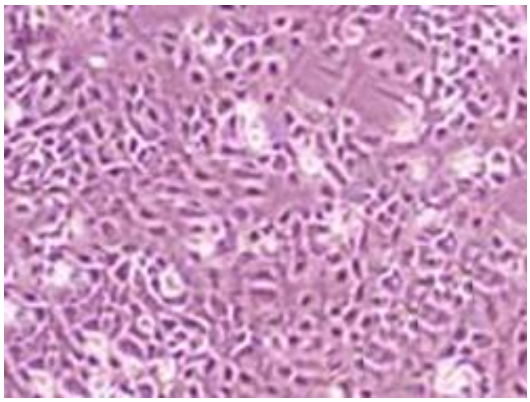
Control



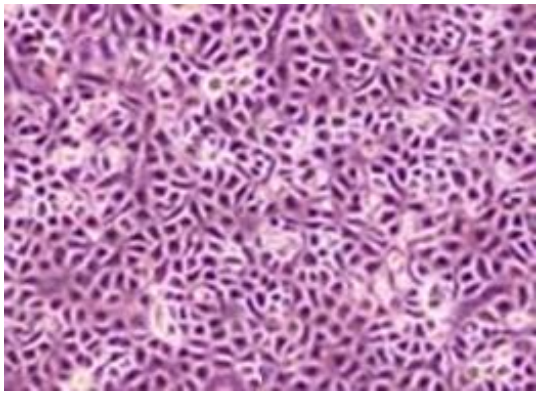
500 µg/ml



250 µg/ml



125 µg/ml



62.5 µg/ml

CONCLUSION

The anti-proliferative activity of MLAG might be due to the presence of a large number of anti-oxidant and anti-proliferative phytochemicals like epithilones, baicaline, Psi, Psi carotene, colchicines, veratramines, vitamin-D-acetate, etc. From the GC-MS analysis it has been found that *A.gangetica* contains many important antioxidant and anti-proliferative compounds. In *"in vitro"* MTT assay the MLAG showed the IC50 value against A549 human lung cancer cell lines and the IC_{50} have been found to be 122.85µg/ml. The cytotoxicity of the MLAG was dose dependent. The anti-proliferative and cytotoxic nature or potency of the plant might be due to the presence of multiple combinations of anti-cancerous compounds.

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