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Cytotoxic and Antiproliferative Activity of Indian Medicinal Plants in Cancer Cells

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Abstract: ***In the present study, six Indian medicinal plants (Pavoniaodorata, Gardenia latifolia, Canthiumdicoccum, Limoniamonophylla, Brideliaroxburghiana and Wrightiatinctoria) are mainly focused for cytotoxicity and anti-proliferating property.Crude methanol extract is obtained for each plant extract using a soxhlet apparatus. 10 mg/ml of extracts of each plant are tested for MTT assay using Calu-6, Colo-205 and HL-60 cells. Based upon the result MTT assay PavoniaodorataandWrightiatinctoria were selected for wound healing assay and cell cycle analysis using Calu-6 cells. Inwound healing assay the migration effect is determined. In case of cell cycle analysis, percentage is determined for the plant- Pavoniaodorata and Wrightiatinctoria. Pavoniaodorata and Wrightiatinctoria showed cytotoxicity and anti-proliferating property for the cancer cell mainly in Calu-6 cells.Hence Pavoniaodorata and Wrightiatinctoria were selected for further assay on the extracts pharmacokinetics and toxicology study should be carried out to support this in vitro assay before in vivo or clinical test is being carried out.***

Keywords: Medicinal plants, Cancer cell lines, MTT assay, Cell Cycle analysis, Wound Healing assay, plant extract preparation.

1. Introduction

Plants have been major source of medicine in all cultures from ancient times. By continuous process of trails and selection, primitive man has learnt to use certain plant juice and crude extracts as antidotes for human disorders. Plants have contributed hugely to Western medicine, through providing ingredients for drugs or having played central role in drug discovery .Some complex diseases, natural products still represent an extremely valuable source for the production of new chemical entities. Most of the natural products found in medicinal plants are the compounds biosynthetically derived from primary metabolites such as amino acids, carbohydrates and fatty acids and categorized secondary metabolites. The roots, seeds, leaves, barks, stems etc may perform specific physiological action in the human body. Alkaloids, compounds of carbon, hydrogen, oxygen and nitrogen, glycosides, essential fatty oils, gums, resins, tannins, mucilage etc are very important substances to use. An abnormal growth of cells which tend to proliferate in an uncontrolled way and to metastasize is called as cancer. The cancer – causing changes in DNA, called mutations can be inherited and may be caused by environmental factors . In Karnataka there would be about 1.5 lakhs cancer cases at any given time and about 35000 new cancer cases are added to this pool each year .Carcinoma ,sarcoma ,leukemia , lymphoma, myeloma , central nervous system cancers are the different types of cancer . Surgery, chemotherapy, radiation therapy, targeted therapy, immunotherapy, hyperthermia, bone marrow and peripheral blood stem cell transplant are the respective treatment procedures. Most drugs intended for cancer therapy are not specific to target cancer cells and may be highly toxic to normal, surrounding tissues.

The existence of traditional medicine depends on plant species diversity and the related knowledge of their use as herbal medicine. In addition both plant species and traditional knowledge are important to the medicine trade and the pharmaceutical industry whereby plants provide raw material and the traditional knowledge perequisite information .Modern research proves the efficacy of some plants such as astragalus, eleutherococcus, shisandra and shiitake mushroom and many of the plants used traditionally in herbalism. *Allium sativum* (Garlic),Aloe barbadencis (Aloe vera), *Beta vulgaris* (Beetroot),*Camellia sinensis* (Green Tea), *Erytroxylum coca* (Coca), *Lycopersiconesculentum* (Tomato), *Ricinuscommunis* (Castor oil), *Vitisvinifera* (Grape) are the particular plants that effect certain types of cancer by improving specific physiological functions .

2. Materials and Methods

2.1 Collection of Plant material

Medicinal plants which belong to the different family are used in the study. Healthy, disease free mature leaves /seeds / rots are collected in and around Bangalore, Karnataka (India) and use for preparation of extract

2.1.1 Selected Plants

Pavoniaodorata (G7PO032)-whole plant part ; *Gardenia latifolia*(G7GL033)- leaves part ;*Canthiumdicoccum* (G7CD041)- leaves part ; *Limoniamonophylla* (G7LM042)-leaves part; *Brideliaroxburghiana* (G7BR048)-whole plant part; Wrightiatinctoria (G7WT049)-leaves part .

2.2 Preparation of extracts

Soxhlet extraction – The desired compound has a limited solubility in a solvent and the impurity is insoluble in that solvent .Soxhletextractor, plant materials, methanol, Whatman filter paper, glass beads are used as materials. According to standard procedure, 10 gms of solid material of a desired plant is weighed and pack in a Whatman filter paper (Thimble).The thimble is loaded in a main chamber of soxhlet and stirrer bar is placed with methanol solvent and 2-3 glass beads . Soxhlet is then equipped with a condenser and solvet is heated to reflux. The desired compound will

dissolve in the warm solvent. When soxhlet chamber is almost full, automatically emptied by siphon side arm, the extract is collected in a still pot. The cycle is repeated for 48 hours. When the desired compound is concentrated in the flask, compound is collected and evaporated to preserve.

2.2.1 Stock solution preparation

10 mg of extract is dissolved in 1 ml of DMSO.

2.3 Biological Assay

2.3.1.1

Cell Lines	*Tissue*	*Morphology*	*Cell type*	*Growth Properties*	*Split Ratio*	*Fluid Renewal*
Calu-6	Lung	Epithelial	Carcinoma	Monolayer	A ration of 1:2 to 1:8	2 to 3 times weekly
HL-60	Blood , peripheral	Lymphoblast	Acute promyelocytic leukemia	Suspension	A ratio of 1:2 to 1:8 is recommended	Every 2 to 3 days
Colo-20	Colon	Round and refractile cells in suspension.	Colorectal adenocarcinoma	Loosely attached and in suspension	Subcultivation ratios of 1:2 to 1:10 arepossible when all cells are pooled	2 to 4 times

2.3.1 Cell Culture

The Human colon adenocarcinoma cell line (Colo -205, semiadherent) and Human acute myelocytic leukemia cell line (HL- 60 , suspension) areobtained from American Type CultureCollection(ATCC). The cells are grown in DMEM containing 2 uM L-glutamine supplemented with 10% fetal bovine serum and 100 U/ml of Penicillin – Streptomycin The cells are incubated at 37^0 C in a humidified 5% CO_2 incubator.

2.3.2 InVitro Assays

2.3.2.1 Cytotoxicity Assay (MTT Assay)

This assay measures the reduction of yellow 3-(4,5-dimethythiazol2-yl)-2,5- diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase.MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product . The cells are then solubilized with an organic solvent (isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically.96- well plate, multi-chennelpipette, cell lines, MEM medium, DMSO, Trypsin, DOX, WST dye are required as materials .According to the standard procedure, the used medium has removed from T-25flask.The cells are trypsinized at 500 rpm for 5 min by adding 2 ml of trypsin. The pallet is resuspended in 2 ml completed media. Cells are diluted to 10,000 cells per ml and use complete media to dilute cells .100 ul of cells are added in to each well. The first two wells were taken as a blank, next two follow by DMSO.DOX is added to next two wells as a standard compound. Plant extracts are added to the next followed wells respectively and incubate for 48 hours. WST dye is added to all wells. After 2 hours incubation, OD at 490 nam is read using ELISA reader.

2.3.2.2 Wound Healing Assay

The Assay is simple, inexpensive and earliest developed method to study directional cell migration in vitro. It mimics cells migration during wound healing in vivo. The basic steps involve creating a “wound” in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. Calu-6 cell line , 24 –well plate ,MEM , micropipette tips, DMSO,DOX,G7PO032,G7WT049 are required for the assay as materials .According to the standard procedure , The cells are seeded in a 24 –well plate (each well contain 1 lakh cells per ml) for 48 hours. The used media is removed from the wells ad using pipette tip a straight scratch is made, simulating a wound .i ml of media is added to all wells except the first one (control). 10 ul of DMSO, DOX, G7PO032 and G7WT049 are added from second well respectively and incubate for 48 hours. The plate is observed under inverted microscopes for every 12 hours.

2.3.2.3 Cell Cycle Assay

Cell cycle analysis is a method in cell biology that employs flow cytometry to distinguish cells in different phases of cell cycle. Before analysis, the cells are permeabilised and treated with a fluorescent dye that stains DNA quantitatively, usually Propidium Iodide (PI). The fluorescence intensity of the stained cells at certain wavelengths will therefore correlate with the amount of DNA they contain. As the DNA content of cells duplicates during the Sphase of the cell cycle, the relative amount of cells in the G_0 phase and G_1 phase (before S phase), in the S phase and the G_2 phase and M phase (after S phase) can be determined, as the fluorescence of the cells in the G_2 / M phase will be twice as high as that of cells in the G_0 /G_1 phase. Cell cycle anomalies can be symptoms for various kinds of cell damage, for example DNA damage, which cause the cell to interrupt the cell cycle at certain checkpoints to prevent transformation into a cancer cell (carcinogenesis). FACS ,96-well plate, micropipette tips, eppendorf tubes ,Calu-6 cell line, DMSO, DOX, PBS, Trypsin ,MEM,G7O032, G7WT049 are required as materials for the cell cycle analysis assay .The cells are seeded in a 96 well plate (20000 cells per well). The compounds G7O032, G7WT049 are tested for different concentration (50ul, 100ul, 200 ul) and incubate for 24 hours . The cultured medium is removed from the wells. 20 ul of trypsin is added to each well .The cells are scraped using pipette tips and then add 100 ul of medium to each well and cells from each well are transferred to eppondorf tube respectively .Then 100 ul of medium is added to each eppendorf tubes and incubate for 5 minutes and centrifuge for 5000 rpm 4^0 C for 10 minutes. The pellet is dissolved in 200 ul of PBS .Then again centrifuge for 5000 rpm 4^0 C for 10 minutes .The pellet is resuspended in 400 ul of PI and transferred to flow cytometer tube and keep in refrigerator for 30 minutes .The result is analysed using flow cytometer .

3. Results

3.1 MTT ASSAY

% viability = (OD of test material / OD of control) X 100
% Inhibition= 100 – (% Viability)

Table 3.1.1

Cytotoxicity assay in HL-60 using WST-1					
Test material (n=2)	Conc^{n} (µg/ml)	OD at 450 nm	Avg. OD	% Viability	% Inhibition
Vehicle control(1% DMSO)	0	1.01 1.01	1.010	100.00	0.0
Dox	100	0.55 0.62	0.585	57.92	42.08
32	100	0.71 0.83	0.770	76.24	23.76
33	100	0.74 0.58	0.660	65.35	34.76
41	100	0.75 0.72	0.735	72.77	27.23
42	100	0.80 0.74	0.770	76.24	23.76
48	100	0.75 0.76	0.755	74.75	25.25
49	100	0.70 0.64	0.670	66.34	33.66

Table-3.1.2

Cytotoxicity assay in Calu-6 using WST-1					
Test material (n=2)	Conc^{n} (µg/ml)	OD at 450 nm	Avg. OD	% Viability	% Inhibition
Vehicle control(1% DMSO)	0	1.67 1.67	1.670	100.00	0.00
Dox	100	0.37 0.39	0.380	22.75	77.20
32	100	0.50 0.44	0.475	28.30	71.70
33	100	0.83 0.52	0.678	40.54	59.46
41	100	0.52 0.54	0.533	31.89	68.11
42	100	0.54 0.59	0.570	34.14	65.86
48	100	0.63 0.73	0.688	41.15	58.85
49	100	0.39 0.46	0.432	25.85	74.15

Table 3.1.3

Cytotoxicity assay in Colo-205 using WST-1					
Test material (n=2)	Conc^{n} (µg/ml)	OD at 450 nm	Avg. OD	% Viability	% Inhibition
Vehicle control (1% DMSO)	0	1.22 2.45	1.842	100	
Dox	100	0.53 0.77	0.657	35.64	64.36
32	100	0.84 0.86	0.858	46.56	53.44
33	100	0.17 0.74	0.462	25.05	74.95
41	100	0 0	0	0	0
42	100	0.51 0.86	0.688	37.34	62.66
48	100	0.76 1.02	0.896	48.63	51.37
49	100	0.52 0.72	0.623	33.81	66.19

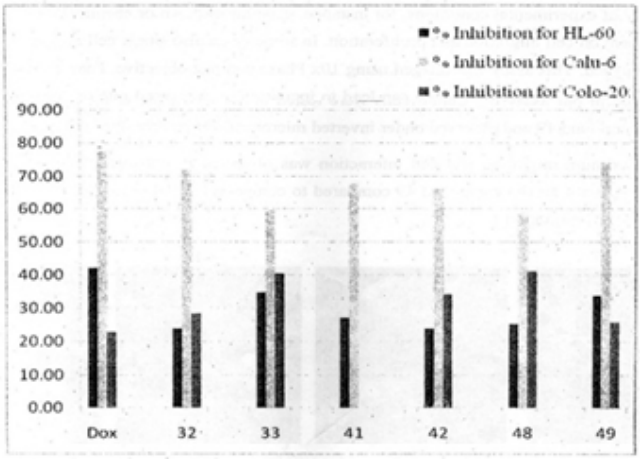

Figure 1: Graph Analysis of different cancer cell lines

Basically, MTT Assay is based on the ability of viable cells with active mitochondrial to produce succinatedehydrogenate enzyme which cleave the tetrazolium rings of MTT where the optical density (OD) obtained is proportional to the number of healthy viable cells. In the study, HL-60 (Table: 3.1.1), Calu-6(Table: 3.1.2) and Colo-205(Table: 3.1.3) are treated with 6 plant extracts at 10 mg/ml concentrations for 48 hours. Based on data collected from experiments in three cell lines anti proliferation effect is evaluated .From the Graph analysis (Fig :1)Percentage of cell inhibition of Calu-6 treated with compound 32 and compound 49 extract indicating that cytotoxic effects are more compared to HL-60 and Colo-205 cell lines.

3.2 Wound Healing Assay

Scratch Wound healing Assay has been widely adapted to study the effects of a variety of experimental conditions for instance, gene – knockdown or chemical compound treatment on cell migration and proliferation. In some cases also single cell migration can be analyzed. This assay is imaged using 10X Phase contrast objective. Factors that alter the motility or growth of the cell can lead to increased or decreased rate of "healing" of the gap .In the assay, the

Calu-6 cells are tested on 24 –well plate using compound 32 and compound 49 and observed under inverted microscope at 10Xfor 0 hour and 48 hours. Maximum migration and cell interaction is observed in untreated, migration rate is moderate for the compound 49 compared to compound 32 however DOX (standard) show 0% migration.

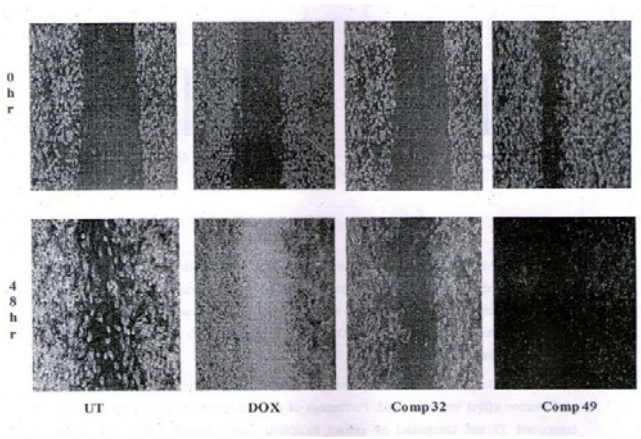

Figure 2: Wound Healing assay analysis

3.3 Cell Cycle Analysis

Flow cytometry is a rapid method to study the cell cycle and DNA content of thousands of individual cells by measurement of light scattering and fluorescene .This method is based on using specific DNA staining dye such as Propidium Iodide (PI), which are applied in the study to monitor physiological status of the cells .In the first part of this study, method routinely used in the in vitro cytotoxicity testing has been applied the MTT test and Wound Healing Assay. The result is further verified by the flow cytometry assay. Cell cycle analysis of drug-treated cells, using flow cytometry, revealed the presence of a distinct cell cycle region below the G_0 / G_1region .This "sub-G_1" peak, displaying particles with lower DNA content, represents apoptotic bodies with their characteristic reduced volume and nuclear condensation. While the percentage of apotosis after exposure to 10 mg/ml of compound 32 and compound 49 for 48 hours is shown (Table:3.3.1) below .More significant increase of apotosis is observed in Calu-6 cells treated with compound 32 and compound 49 .

Table 3.3.1

	% Gated			
Marker	UT	DOX	Comp32	Comp 49
All	100	100	100	100
G_0/G_1	79.18	35.34	27.62	47.02
G_2-M	3.95	15.43	26.02	7.59
S	13.96	62.69	32.64	32.90

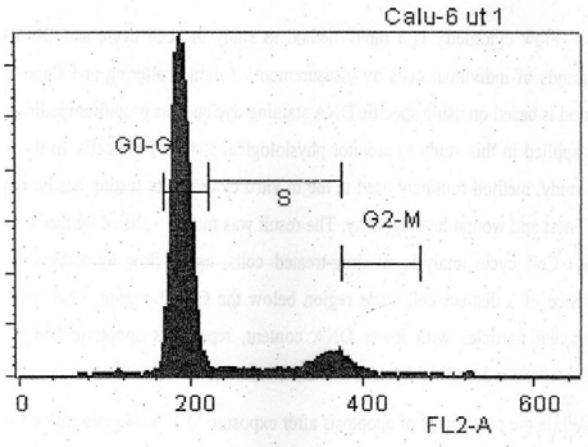

Figure 2: UT

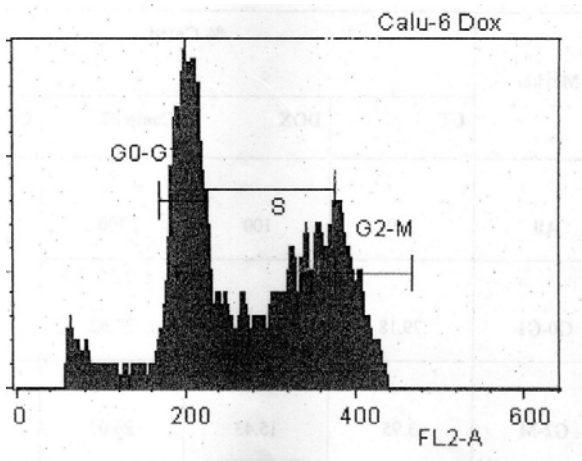

Figure 3: DOX

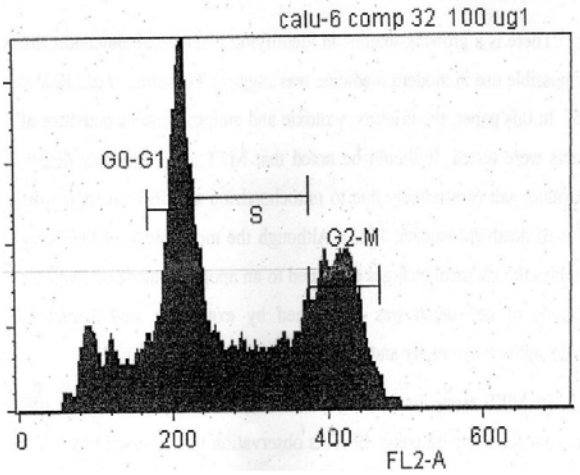

Figure 4: Comp 32

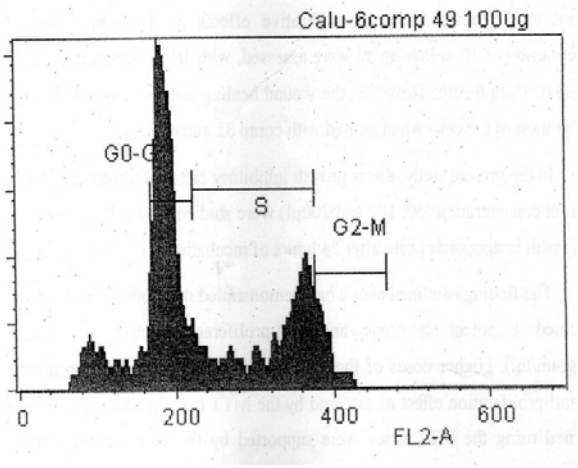

Figure 5: Comp 49

4. Discussion

There is a growing interest in identifying plant-based anticancer drugs ever since their possible use in modern medicine was suggested. In this paper, the in vitro cytotoxic and anti-proliferation activities of the six plant extracts were tested. It should be noted that MTT assay can only detect formation of tetrazolium salt (presumably due to mitochondrion activity) but cannot differentiate the modes of death. Although the morphology of the dying cells closely resembles the classical changes attributed to an apoptotic mode of death. For this reason, the mode of cell death was determined by examining well-characterized apoptosis markers by flow cytometry and by wound healing assay.

In MTT assay the absorbance reading after 48 hours showed more significant effect on comp 32 and comp 49. This observation was supported by a study that shows cytotoxic and anti-tumour activities of *Pavoniaodorata* against Erlich's ascites carcinoma cells bearing mice and studies of antiviral activity and cytotoxicity of Wrightiatinctoria and *Morindacitrifolia* .The two extracts used showed a cytotoxic effect on Calu-6, Colo-205 and HL60 cells. In an attempt to determine the anti-proliferative effects of the extract tested, concentration of 10 milligram/ml of dox as positive control on Calu-6 cells was used. However, the wound healing assay test showed lower percentage of migration of the cells when treated with comp 32 and comp 49.
In the present study, direct growth inhibitory effect of the comp 32 and comp 49 at different concentrations (50, 100 and 200µl) were studied. The cell cycle analysis revealed good result in apoptosis peak, after 24 hours of incubation for 100µl concentration.

The findings outlined above had demonstrated that both the comp 32 and comp 49 possessed a potent cytotoxic and anti-proliferation action at concentration (10 milligram/ml). Higher doses of the extracts were found to exhibit pronounce cytotoxic and anti-proliferation effect as assessed by the MTT reading. However, cytotoxic indexes obtained using the MTT assay were supported by the results those obtained with the wound healing assay and cell cycle analysis at which the migration and viability of cells moderate dramatically for both extracts at 10 milligram/ml.

However, in wound healing assay test showed lower percentage of migration of cells for the comp 32 and comp 49 for 10 milligram/ml. In case of cell cycle analysis percentage of apoptosis was more for comp 32 compared to comp 49.

Comp 32 and Comp 49 showed more promising anti-proliferative and apoptotic activity against HL-60, Calu-6 and Colo-205 cells, compared to Comp 33, Comp 41, Comp 42 and Comp 49. Besides, the inhibition of proliferation of Calu-6 was roughly constant over a wide concentration range (25-100 milligram/ml) for Comp 32 and Comp 49. Since extracts which regulate apoptosis and overcome apoptosis deficiency of cancer cells are of high medical significance, further chemical and biochemical studies are currently under way.

This current study has effectively that these Indian medicinal plants have a good anti-leukemia potential with less or no toxic effects towards healthy immune system. Comp 32 and Comp 49 methanol extract have been successfully exhibited to be cytotoxic towards Calu-6 cell lines. Further assay on the extract's pharmacokinetics and toxicology study should be carried out to support this in vitro assay before in vivo or clinical test is being carried out.

5. Conclusion

Identification andselection of 6 Indian medicinal plants for anticancer properties. Optimization of extraction method for active from Indian medicinal plants for anticancer properties. Functional assays for the anticancer properties of different solvent extract/active principles by invitro cell based assy. Cell proliferation and cell distribution studies using flow cytometry from active extracts/compounds.

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