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ANTICANCER ACTIVITY OF SOME MEDICINAL PLANTS FROM HIGH ALTITUDE EVERGREEN ELEMENTS OF INDIAN WESTERN GHATS

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Abstract : Traditional medicine has a long history of serving peoples all over the world. India is without doubt a herbal hub. Medicinal plants that are native to India and their use in various traditional systems of medicine are indeed awe-inspiring. The ethnobotany and ubiquitous plants provide a rich resource for natural drug research and development. In recent years, the use of traditional medicine information on plant research received considerable interest. In the present study, twenty one plant extracts of different species used by Indian traditional healers for the treatment of ulcers, cancers, tumors, warts, and other diseases, were tested *in vitro* for their potential anticancer (antiproliferative and cytotoxic) activity. The ethanolic extracts were tested against six human cancer cell lines using MTT 3-(4,5-dimethylthiazol–2-yl)- 2,5-diphenyltetrazolium bromide. colorimetric assay and the cytotoxicity was confirmed by Soft agar colony forming 'Clonogenic assay'. Seven out of the 21 extracts (33%) showed remarkable cytotoxic potential. The highest activity was found in the leaf/stem ethanol extracts from *Plectranthus urticioides* and *Garcinea morella* against all the six human cancer cell lines screened. The present finding may pave the way for the bioactivity guided fractionation and isolation of important moieties for the anticancer chemotherapy and in part help to minimize the serious lacuna in scientific validation of these herbs.

Keywords: Ayurvedic medicine,Traditional medicine, Anticancer, MTT, Clonogenic, *Plectranthus urticioides, Garcinia morella.*

Awareness about natural sources is world wide on rise and in the context, and then novel bio molecules from natural products (plant sources) offer a great scope.[1] India being one of the 12 major diversities centers, hold large reservoir of plant genetic diversity, which can provide novel biomolecules.

Ayurveda, Siddha and Unani systems of medicines offer a good base for scientific exploration of medicinally important molecules from nature. The rediscovery of Ayurveda is a sense of redefining it in modern medicines. Emerging concept of combining Ayurveda with advanced drug discovery programme is globally acceptable. Traditional medicine has a long history of serving peoples all over the world. Medicinal plant is an important element of indigenous medical systems in China as well as elsewhere. The ethnobotany and ubiquitous plants provide a rich resource for natural drug research and development. In recent years, the

use of traditional medicine information on plant research has again received considerable interest. While the Western use of such information has also come under increasing scrutiny and the national and indigenous rights on these resources have become acknowledged by most academic and industrial researchers. Meanwhile, the need for basic scientific investigations on medicinal plants using indigenous medical systems becomes imminent. Most recently emerging approaches such as advanced genomics has significantly facilitated the lead compounds for drug development, high throughput screening combinatorial chemistry with biology and computer assisted *de novo* drug design. The reason why plants are best suited for insulating novel chemical entities arises from the fact that plant molecules have always shown compatibility through natural resistance or tolerance against them because of that approximately 60%of the anticancer and anti infective agents that are commercially available are of natural product origin.[2] There seems to be increasing possibility of finding biological activity among plants with recorded medicinal uses rather than from plants randomly selected.[3,4]

Furthermore, selection of plants gives better criteria for screening programs especially in its initial phases, compared to the screening of compounds isolated and/or purified from natural products.[5,6] Finally, the strategy for research and *in vitro* evaluation of biological activity of natural products has changed in the past few years. One of the recent developments is the highly automated bioassay screening method based on colorimetric assays, which quantify the proliferation of cell cultures.[7,8] These techniques, considered rapid and inexpensive for the evaluation of antitumour[9,10] and antiviral activity[11]of a large number of natural products, have also permitted the isolation and purification of biologically active principles.[4,6]

The objective of our work was to evaluate *in vitro* antiproliferative and/or cytotoxic activity of some plant species known to have traditional medical uses against skin infections including ulcer, tumor and cancer etc. using colorimetric assays. In our *in vitro* anticancer screening program, we routinely use 3 assays for the evaluation of extracts of natural products with possible anticancer activity. One of these methods is based on the metabolic reduction of MTT[9] and the other is clonogenic assay[12] based on the concept that each single cell has a proliferative potential to renew its population with a limited number of potential division. Both MTT and clonogenic assay gives the antiproliferative and cytotoxic potential of a drug provided to the cancerous cells. Even though a most potent antiproliferative and cytotoxic extract found in MTT assay and clonogenic assay cannot be used for further drug formulations as they also affect the uninfected cells too for which the Bacopa sensor test[13] is used as a standard assay for the evaluation of the cytotoxicity caused by the extracts if any on the normal cells of the system.

The selection of the plant species for the present study was mainly based on the traditional uses of these species for the treatment of various diseases including skin infections, ulcer, tumor, cancer etc. The *in vitro* anticancer activity of ethanol, water and hydro-alcoholic extracts of 21 plant species has been studied in this study. A preliminary evaluation of the potential cytotoxic activity of all these extracts was carried out using the MTT method (**Table 1**). Out of the twenty one plant extracts screened 7 showed potential results. These identified 7 plant extracts were confirmed for their cytotoxic potentials by clonogenic assay (**Table 2**). The cytotoxic doses of the 7 extracts along with their 2 fold higher concentrations were then analyzed by Bacopa Sensor test to test the viability of the explants (Data not shown).

Table 1. Plant extracts screened for their antiproliferative activity by MTT assay

	PLANT NAME	PARTS USED	KB		MCF-7		COLO		CaCO2		PA-1		WRL-68	
S. No			IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90	IC-50	IC-90
1	*Eucalyptus .sp*	Leaf	40	70	20	80	IA	IA	IA	IA	60	50	40	100
2	*Clausena. sp*	Leaf & Fruits	37	100	54	80	IA	IA	IA	IA	50	40	25	75
3	*Trichopus zeylanicus*	Leaf	35	98	46	75	IA	IA	IA	IA	100	60	40	IA
4	*Aleurites molucana*	Fruits	IA	IA	90	IA	IA	IA	IA	IA	IA	IA	IA	IA
5	*Garcinea cambogea*	Leaf	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA
6	*Begonea floccifera*	Leaf	25	60	50	85	50	100	60	95	45	55	35	70
7	*Gynura pseudo china*	Leaf & Stem	25	55	95	IA	65	IA	25	55	45	55	60	IA
8	*Decalebis arayalpathra*	Leaf & Stem	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	40	100
9	*Erythropalum populifolium*	Leaf	45	80	IA	IA	IA	IA	IA	IA	6.0	8.0	IA	IA
10	*Plectranthus urticoides*	Leaf & Stem	**6**	**15**	**55**	**90**	70	IA	IA	IA	**1.0**	**0.8**	**6.0**	**25**
11	*Garcinea morella*	Leaf	**1**	**4**	**40**	**65**	**10**	**40**	**3.0**	**8.0**	**0.9**	**2.5**	**8.0**	**40**
12	*Justica beddomeoo*	Leaf & Stem	IA	IA	IA	IA	10	IA	100	IA	IA	IA	IA	IA
13	*ArInychia pedunculata*	Leaf	25	50	40	80	**4.8**	30	20	50	20	50	10	65
14	*Erythropalum populifolium*	Stem	100	IA	IA	IA	100	IA	IA	IA	IA	IA	IA	IA
15	*Schefflera racemosa*	Leaf & Stem	25	65	7.5	40	10	60	45	85	60	IA	4.0	20
16	*Cissus quadragularis*	Stem	IA	IA	90	IA	IA	IA	IA	IA	IA	IA	IA	IA
17	*Heradeum sp.*	Leaf	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA
18	*adenia hondala*	Tuber	IA	IA	IA	IA	18	IA	IA	IA	IA	IA	IA	IA
19	*Aristolochia tagala*	Leaf & Stem	9	40	40	75	70	IA	IA	IA	13	25	20	60
20	*Decalepis arayalpathra*	Tuber	IA	IA	7.5	9.0	100	IA	20.0	60.0	40.0	80.0	10.0	40.0
21	*Millettia rubiginosa*	Stem	IA	IA	75	IA	60	IA	IA	IA	IA	IA	15	IA
22	*Taxol*	Pure	0.001	9.0	0.1	2.5	0.01	0.8	0.007	1.25	0.017	2.0	0.7	7.5

As can be seen in **Table 1 and 2**, the ethanol extract of tubers of *Decalebis arayalpatra* showed potential antiproliferative and cytotoxicity against all the screened cell lines, except KB and Colo. While the ethanol extract of the leaves of *Decalebis arayalpatra* was found to exhibit no inhibition of growth even at higher doses. This confirms that the tuber of *Decalebis arayalpatra* possess the active moiety supplementing antiproliferative and/or cytotoxic property.

In the case of *Erythropalum populifolium*, the leaf ethanolic extract possessed cytotoxic property against PA-1, KB and WRL, while the ethanolic stem extract showed no cytotoxic effect against any of the six cancer cell line used.

Secondly, the ethanol extract of leaf of *Garcinea morella* represented high cytotoxic values of 1.0 and 4.0 (KB-403), 8.0 and 40.0 (WRL-68), 3.0 and 8.0 (CaCO 2), 0.9 and 2.5 μg/ml (PA-1) as

Table 2. Cytotoxic effects of different plant extracts by Clonogenic assay (µg/ml)

	NAME	Part Used	KB		MCF-7		COLO 320 DM		CaCO2		PA-1		WRL-68	
Sr. No.			IC-50	IC-90	IC-50	IC-90	IC-50	IC-90	IC-50	IC-90	IC-50	IC-90	IC-50	IC-90
1	*Plectranthus urticoides*	Leaf & Stem	**3.31**	**35.48**	**56.23**	**100**	**100**	**IA**	**IA**	**IA**	**0.398**	**6.31**	**7.08**	**52.48**
2	*Garcinea morella*	Leaf	**3.981**	**33.11**	**31.62**	**IA**	**4.47**	**100**	**0.063**	**35.48**	**5.01**	**35.48**	**6.31**	**63.09**
3	*Schefflera racemosa*	Leaf & Stem	19.95	100	5.62	50.12	12.02	50.12	50.11	IA	15.84	100	12.58	79.43
4	*Erythropalum populifolium*	Leaf	52.48	IA	IA	IA	IA	IA	IA	IA	1.78	17.78	7.24	52.48
5	*Acronychia pedunculata*	Leaf	19.95	87.09	52.48	100	**5.01**	63.09	17.78	100	15.84	100	25.11	79.43
6	*Aristolochia tagala*	Leaf & Stem	12.0	85.6	58.5	95.4	IA	IA	IA	IA	24.6	45.4	32.5	85.7
7	*Decalepis arayalpathra*	Tuber	IA	IA	19.95	25.11	IA	IA	25.11	79.43	63.09	100	25.11	52.48
	Taxol	Pure	0.005	17.78	1.0	4.0	0.04	4.47	0.01	5.01	0.063	5.01	1.78	15.84

50% and 90% inhibitory concentrations on the cell growth. But ethanol extract of leaf of *Garcinea cambogea* was found inactive against all the six cell lines tested. This shows that there is some intra specific variation in the chemical constituents of the two genus of the same species of *Garcinea* and thus *Garcinea morella* can be said to possess the additional molecule (s) in the leaf providing antiproliferative, cytotoxic and anticancer properties.

Finally in Bacopa sensor test all extracts except *Achronychia pedunculata* represented no morphological variations as compared to control, while the presence of ethanol extract of the leaf of *Achronychia pedunculata* in the media, restricted the growth of the explant of *Bacopa monnieri* and the explant died in 15 days due to the cytotoxicity. Therefore in this study we have identified six potential anticancer plant extracts which when further subjected to bioactivity guided fractionation will provide some important moieties for the anticancer chemotherapy.

Experimental

Plant collection

Different plant species based on their traditional usage were collected from the sub-tropical region of the Western ghats of India. Species like *Plectranthus* and *Achronychia* were collected from the high altitude of 1500 m while rests of the species were collected from 800-1000 m altitude.

Extract preparation

200 g of plant tissue obtained from different parts (leaf, stem, root, tuber, bark etc.) of the plants described in **Table 1** were shade dried at ambient temperature and powdered. The powdered plant material was percolated with 500 ml of the following solvents: ethanol and water. Additionally, water-ethanol (50:50) extracts were also prepared from dried powdered plant materials. The various extracts were carefully evaporated to dryness under reduced pressure (Buchi Rotavapor) and were stored at – 20° C, until used. To test the biological activity, the dried crude extracts were dissolved in Dimethyl Sulphoxide (DMSO, Sigma) to a concentration of 100 mg/ml, and at the time of assay two-fold dilutions were prepared in cell culture medium starting from a concentration of 1,000 µg/ml of extract.

Cell culture

The representative cell lines used were *CaCO2 and Colo-320-DM* human colon cancer cell line (ATCC HTB-37 and CCL 220 respectively), KB human oral cancer cell line (ATCC CCL-17), PA-1 human ovary cancer cell line (ATCC CRL 1572), MCF-7 human breast cancer cell line (ATCC HTB 22), WRL-68 human liver cancer cell line (ATCC CL-

48).The cryopreserved cells were revived from liquid nitrogen using heat shock method and centrifuged twice at 1000 RPM for 10 minutes to remove the DMSO used during Cryopreservation and cultured in 25 cm^2 flasks. Once the cells had covered about 80% of the surface, they were trypsinized and the titre was adjusted using haematocytometer for antiproliferative and cytotoxicity measurements as described by **Absher (1973)**. All cells were grown in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin and 0.07% $NaHCO_3$. The cultures were maintained at 37°C in humidified 5% CO_2 atmosphere.

MTT assay (Antiproliferative assay)

Cell monolayers were trypsinized, washed with culture medium and plated in a 96-well flat-bottomed plate with $5x10^3$ cells per well for CaCO2, Colo-320-DM and MCF-7 cells, while $2x10^4$ cells per well for PA-1 cells and $5x10^4$ for WRL-68 and KB-403 cells. After 24 h incubation, each diluted extract was added to the appropriate wells and the plates were incubated for a further 4 h at 37°C in a humidified incubator with 5% CO_2. The supernatants were removed from the wells and cells were incubated for their respective doubling times (average 48 h).After the incubation each well was added with 10ìl of (5mg/ml in PBS) MTT and were incubated again for 4 h. Cell viability was evaluated by the MTT colorimetric technique.[9]

Furthermore all tests were compared with a positive control Taxol (Sigma) and a negative control (untreated cells) and tested simultaneously under identical conditions. The results are obtained from triplicate assays with at least 5 extract concentrations and are expressed as the mean obtained from 3 different assays. The percentage of antiproliferative and / or cytotoxic activity is calculated as (A-B)/A. x 100, where A and B are the OD_{570} of untreated and of treated cells, respectively.

Clonogenic assay (Cytotoxicity assay)

The extracts representing potential activity in MTT were confirmed by Soft Agar Colony Forming Assay as described by **Albert *et al.* (1980)** for their cytotoxicity. Briefly, base agar was prepared by mixing 1% molecular biology grade agar with 2X growth media in 12 well polystyrene micro plates. Cells mixed to 0.7% agar and 2X growth media were suspended over base agar along with the different doses of extracts. The assembly was incubated at 37^0C, 5% CO_2 and 95% atmosphere for 15 days. The colonies consisting of more than 40 cells were scored on day 14 using dissecting microscope. Taking untreated cells as control the inhibition was calculated as IC-50 and IC-90 values of the compound that is 50% and 90% inhibition concentration respectively and was calculated as a result of three assays in triplicate with at least 5 concentrations.

Bacopa sensor test

We screened 21 plant extracts out of which 7 showed potential antiproliferative and cytotoxic activity in MTT and Clonogenic assay. These extracts were further subjected to Bacopa sensor test to assess the effect of cytotoxic extract on the normal growing cells taking *Bacopa monnieri* plant *in vitro* as a biosensor as established by **Khanuja *et al.* (1998)**. The explants having two nodes and one internode were inoculated in MS having no or zero growth hormone. Different doses of extracts (100ìg, 10ìg, 1ìg and 0.1ìg/ml) were mixed with the media and were inoculated with the explants for 20 days.

Morphological variations were recorded everyday taking the untreated explant as control. Each extract dilution was added in 7 replicates and the mean was taken as the actual

readings.The results were also compared with Taxol (+control) and untreated explant (-control).

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