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In-Vitro Anticancer Activity of Standard Extracts Used In Ayurveda

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ABSTRACT

The hydro-alcoholic extracts of five Ayurvedic medicinal plants, pericarp of Terminalia chebula, rhizome of Acorus calamus, stem bark of Bauhinia variegate, whole plant of Phyllanthus amarus, root of Glycyrrhiza glabra were evaluated for their antiproliferative activity on fourteen cancer cell lines. These plant extracts were tested by sulforhodamine-B (SRB) assay for its anti proliferative activity and four extracts except Glycyrrhiza glabra were found active against prostrate cancer cell line (DU145. In addition to this Terminalia chebula exhibited activity against leukemia cancer cell line (K562).

Keywords: Standardization, Anticancer, Cell Line, SRB Assay.

INTRODUCTION

The practice of herbal medicine dates back to the very early periods of known human history. There is evidence of herbs having been used in the treatment of diseases and for revitalizing body systems in almost all ancient civilizations such as the Indian, the Egyptian, the Chinese, the Greek and the Roman civilizations. Plants were the main stem of medicine and credited with mystical and almost supernatural powers of healing. Plants have a vast potential for their use as curative medicine. In the recent times the trend in the research in the field of cancer is once again shifting towards identifying new medicines for the treatment of cancer from natural sources. Wide varieties of compounds are in use in current cancer therapeutic practices. Chemical adjuvants of bacterial origin such as Bacillus Calmette Guerin (BCG) have been shown to exert therapeutic effects in the treatment of cancer. But, the effect is limited due to number of undesirable side effects [1]. Agents such as levamisole and interferons were widely used to treat cancers in the mid 1970s to early 1980s [2].

However, no study on the standardized hydro-alcoholic extracts of plants has been reported. In order to verify the anecdotal claims, we have investigated the anticancer activity of these plant extracts by using different cell lines.

MATERIAL AND METHODS

Drug procurement and authentication

Botanically identified and authenticated raw plant materials of fruit pericarp of *Terminalia chebula*, rhizome of *Acorus calamus*, stem bark of *Bauhinia variegate*, whole plant of *Phyllanthus amarus*, root *of Glycyrrhiza glabra* were procured from medicinal plants gardens of amalgamated units of Central Council for Research in Ayurveda and Siddha and the raw materials were subjected to hydro-alcoholic extract preparation.

Preparation of Hydro-alcoholic extract (60: 40) and Standardization

40 % ethanol was added to the coarse powdered samples in a ratio of 4:1 and macerated the mixture for four hours. Then the mixtures were heated for 2 hrs at 80^0 C and the steps were repeated 3 times. Each time sufficient quantity of 40 % ethanol was added when required. Then the extract was filtered and concentrated under vacuum. After vacuum tray drying at 70–80°C for 14–16 hrs the dried lumps were milled up to the particle size of 40 mesh. The extracts were analyzed and standardized for their Physico-chemical parameters along with heavy metal analysis and microbial contamination as per API / WHO guidelines.

SRB Assay[3]

The anticancer activities of extracts were studied at Advanced Center for Treatment, Research and Education in Cancer (ACTREC), Mumbai where 14 cell lines were maintained in ideal laboratory conditions. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates 90 µL/well at appropriate plating densities, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, in 5% CO_2, 95% air and 100 % relative humidity for 24 h prior to addition of experimental drugs.

Table 1: List of Different Cell Lines used for Experiment

Sr. No.	Name of the Cell Line	Human Tissue of Origin
1	Colo205	Colon
2	Hop62	Lung
3	HT29	Colon
4	SiHa	Cervix
5	MIAPACA 2	Pancreas
6	DWD	Oral
7	T24	Bladder
8	PC3	Prostate
9	A549	Lung
10	ZR-75-1	Breast
11	A2780	Ovary
12	DU145	Prostate
13	MCF7	Breast
14	K562	Leukemia

After 24 h, cells from one plate of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental extracts were solubilized in appropriate solvent at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 10 times the desired final maximum test concentration with complete medium containing test article at a concentration of 100, 200, 400 and 800 µg/ml. Aliquots of 10 µl of these different dilutions were added to the appropriate micro-titer wells already containing 90 µl of cell suspension, resulting in the required final drug concentrations of 10, 20, 40 and 80 µg/ml.

Table 2: Standardization of hydro-alcoholic extract of samples

S. No.	Parameter	Values				
1.	Sanskrit Name	*Acorus calamus* (Rhizome)	*Bauhinia variegate* (Stem bark)	*Terminalia chebula* (Fruit pericarp)	*Phyllanthus amarus* (Whole plant)	*Glycyrrhiza glabra* (Root)
2.	Loss on Drying	4.94 %	4.99 %	5.03 %	6.37 %	3.53%
3.	Water soluble extractives	83.0 %	55.93 %	78.84%	78.10 %	88.54%
4.	Alcohol soluble extractives	43.55 %	35.44 %	98.28%	51.75 %	64.93%
5.	pH	3.63	5.58	3.24	4.51	5.52
6.	Total ash	14.09 %	7.31 %	3.63 %	14.75 %	7.09%
7.	Acid Insoluble Ash	1.09 %	1.62 %	0.59%	1.90 %	2.60%
8.	Bulk Density (gm /ml)	0.51	0.49	0.67	0.72	0.73
9.	Tapped Density (gm /ml)	0.85	0.69	1.1	0.84	1.04
10.	Heavy metal anaysis (By AAS)					
	a) Lead	<5ppm	<5ppm	<5ppm	<5ppm	<5ppm
	b) Cadmium	<1ppm	<1ppm	<1ppm	<1ppm	<1ppm
	c) Arsenic	<2ppm	<2ppm	<2ppm	<2ppm	<2ppm
11.	Assay	–	–	75.87 % Tannins on d/b (volumetric method)	12.06 % Bitters on d/b (gravimetric method)	13.92 % Glycyrrhizic acid on d/b HPLC method
12.	Microbiological Limits					
	Total Plate Count	400 Cfu/gm	500 Cfu/gm	100 Cfu/gm	200 Cfu/gm	400Cfu/gm
	Yeast/Molds	<10 Cfu/gm	40 Cfu/gm	<10 Cfu/gm	<10 Cfu/gm	<10Cfu/gm
	Enterobacteriaceae	Absent	Absent	Absent	Absent	Absent
	E.Coli	Absent	Absent	Absent	Absent	Absent
	Salmonella	Absent	Absent	Absent	Absent	Absent
	Staphylococcus aureus	Absent	Absent	Absent	Absent	Absent
	Pseudomonas aeruginosa	Absent	Absent	Absent	Absent	Absent

Table 3: Median Growth Inhibition (GI_{50}) values for test extracts (µg/ml); (yellow highlighted tests indicate activity)

	Median Growth Inhibition (LC_{50}) values for test extracts (µg/ml)													
	Colon	Lung	Colon	Cervix	Pancreas	Oral	Bladder	Prostate	Lung	Breast	Ovary	Prostate	Breast	Leukemia
Name of the Drug	**Colo205**	**Hop62**	**HT29**	**SiHa**	**MIA-PA-CA-2**	**DWD**	**T24**	**PC3**	**A549**	**ZR-75-1**	**A2780**	**DU145**	**MCF7**	**K562**
Acorus calamus	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	13	>80	>80
Bauhinia variegate	>80	>80	>80	>80	28	>80	>80	>80	>80	>80	>80	<10	>80	54
Terminalia chebula	>80	60	79	>80	>80	76	51	48	>80	48	21	<10	31	<10
Phyllanthus amarus	>80	77	>80	>80	>80	79	>80	67	>80	>80	26	<10	31	24
Glycirrhyza glabra	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	73	>80
Doxorubicin Positive Control	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	10	<10	<10

Table 4: Total growth Inhibition values (TGI) (µg/ml)

	Colon	Lung	Colon	Cervix	Pancreas	Oral	Bladder	Prostate	Lung	Breast	Ovary	Prostate	Breast	Leukemia
Name of the Drug	**Colo-205**	**Hop-62**	**HT-29**	**SiHa**	**MIA-PA-CA -2**	**DWD**	**T24**	**PC3**	**A-549**	**ZR-75-1**	**A-2780**	**DU-145**	**MCF7**	**K562**
Acorus calamus	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
Bauhinia variegate	>80	>80	>80	>80	51	>80	>80	>80	>80	>80	>80	52	>80	>80
Terminalia chebula	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	48	29	62	27
Phyllanthus amarus	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	50	41	60	62
Glycirrhyza glabra	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
Doxorubicin, Positive control	<10	15	51	11	18	<10	43	10	<10	30	<10	35	26	38

Table 5:Lethal concentration values (LC_{50}) (µg/ml)

Name of the Drug	Colo205	Hop62	HT29	SiHa	MIA-PA-CA -2	DWD	T24	PC3	A549	ZR-75-1	A-2780	DU-145	MCF7	K562
Acorus calamus	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
Bauhinia variegate	>80	>80	>80	>80	74	>80	>80	>80	>80	>80	>80	>80	>80	>80
Terminalia chebula	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	75	60	>80	58
Phyllanthus amarus	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	74	77	>80	>80
Glycirrhyza glabra	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
Doxorubicin, Positive control	38	48	>80	49	50	42	>80	44	37	65	39	57	59	67

For each of the experiments a known anticancer drug was used as a positive control.

Endpoint Measurement

After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM Trizma base, and the absorbance was read on an Elisa Plate Reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100.

Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)]; the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:

[(Ti−Tz)/(C−Tz)] × 100 for concentrations for which Ti>/=Tz (Ti−Tz) positive or zero [(Ti−Tz)/Tz] × 100 for concentrations for which Ti<Tz. (Ti−Tz) negative.

The dose response parameters were calculated for each test article. Growth inhibition of 50 % (GI50) was calculated from [(Ti−Tz)/(C−Tz)] × 100 = 50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti−Tz)/Tz] × 100 = −50.

Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested. The summary of parameters is as follows.

GI50 Growth inhibition of 50 % (GI50) calculated from [(Ti−Tz)/(C−Tz)] × 100 = 50, drug concentration resulting in a 50% reduction in the net protein increase

TGI Drug concentration resulting in total growth inhibition (TGI) will calculated from Ti = Tz

LC50 Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti−Tz)/Tz] × 100 = 7minus;50.

GI50 value of ≤ 20 µg/ml is considered to demonstrate activity.

RESULTS

The standardization of hydro-alcoholic extracts (Table 2) of plant materials has been carried out as per the standard guidelines and the anticancer activity of extracts was conducted against fourteen different cell lines of which four plants extract have shown the anti-cancer activity against prostrate (DU145) cell lines (Table 3). In addition, *Terminalia chebula* was active against leukemia cell line whereas *Glycyrrhiza glabra* has not shown activity against any of the 14 cell lines used for screening.

DISCUSSION AND CONCLUSION

We have selected five plant extracts for studying anti-cancer activity based on available literature and found four plants having activity against prostrate cancer cell line DU 145. With GI_{50} values 13, 10, 10 and 10 in *Acorus Calamus, Bauhinia variegate, Terminalia chebula*, and *Phyllanthus amarus*, respectively. *Glycerrhiza glabra* has not shown activity against any cell-line. Present study implicit the observation that *Glycerrhiza glabra* is not a promising anti-cancer plant against the selected cell line. However, *Terminalia chebula* was active against leukemia cell line (K562) at LC_{50} less than 10µg/ml analogous to prostrate cancer cell line. Apparently, the promising active principle in *Terminalia chebula* inhibits both prostrate cancer and leukemia indicating need to investigate underlying mechanism by which this activity was exhibited. Further, all these plant-extracts need to be screened against different cell lines apart from the selected cell lines to confirm the activity.

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