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Antiproliferative bioassay of extremophilic medicinal plants from Langtang Himalayan range of Nepal

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Abstract The seven different high altitude medicinal plants viz. *Genetiana depressa, Rhododendron setosum, Rhodiola spp., Elsholtzia strobilifera, Hedychium spicatum, Eriophyton wallichii, Rheum spp.,* were chosen as plant samples. With light of results from Thin Layer Chromatographic analysis, cold methanol was used as a solvent for extraction process. Brine Shrimp bioassay was performed to find the cytotoxic effect (LC_{50}) of the extract at various concentrations at interval of 24 and 48 hours. Anti-proliferative effect of the extract (IC_{50}) was observed against HeLa cancer cell line by performing the MTT assay. The results of this study compel us to suggest that these plants extracts can be a promising drug candidate in the need for more effective anticancer agents.

Keywords: *Genetiana depressa, Rhododendron setosum, Rhodiola spp., Elsholtzia strobilifera, Hedychium spicatum, Eriophyton wallichii, Rheum spp.,* LC_{50}, IC_{50}, HeLa cell line, Thin Layer Chromatography, Anticancer.

INTRODUCTION

Plants with medicinal value offer us new sources of drugs which have been used for centuries in traditional medicine. There are many compounds used in medicines today which are derived from plants. Undoubtedly, plant kingdom has many plants containing substances of medicinal value which are yet to be discovered (WHO Monographs on Selected Medicinal Plants Vol. 1, 1999). The traditional health care system based on Himalayan native plants has a long history. Among MAPs, some species are used for medicinal purposes, some for the aromatic purposes and many for both medicinal and aromatic purposes (Malla *et al*, 1997). MAPs are widely used in Nepal as medicine, additives, beverages, cosmetics, sweeteners, bitters, spices, dyeing agents and insecticides. Currently, research and development of new drugs from natural resources in a systematic and strategic manner has become the global trend. Natural products derived medicines account for 30% of therapeutic agent presently prescribed in clinic. Plant originated natural products have played and will continue to play important role in pharmaceutical industry to discover and deliver chemicals and biological entities for the treatment of various diseases (Gupta, 1994).

MATERIALS AND METHODS

Collection of plant samples

The seven different high altitude medicinal plants viz. *Genetiana depressa, Rhododendron setosum, Rhodiola spp., Elsholtzia strobilifera, Hedychium spicatum, Eriophyton wallichii, Rheum spp.,* were chosen as plant samples. All plants were identified by expert botanists from Kathmandu University and National Botanical Garden, Nepal. The plant samples were collected from Gosaikunda (4360 m) to Suryakunda (4800 m) region of Langtang, Rasuwa situated in Bagmati zone of Nepal with the Latitude: 28° 13' 0' N and Longitude: 85° 34' 60' E.

Extraction

The plant samples were dried and then crushed in grinder to form the powder. 1 gram of crushed sample was added to 100 ml of methanol solvent, just to cover the upper layer of sample and was shaken regularly .After 24 hrs, filtration was done and fresh methanol was again added followed by storing of filtrate. The number of extraction was repeated and determined by visualizing the color of the filtrate. Finally, the collected filtrate was vaporized using a rotavapor® (Bucci) and water bath at 40°C, and the final extract was weighed to calculate the yield.

Brine Shrimp Assay

Cleaned test tubes were divided into four groups each group consisting of five test tubes. After 24 hrs of incubation, the nauplii were recovered with a pipette and 10 nauplii were transferred in each test tube.The groups were administered with different dilutions of sample. The test tubes were then incubated at 32 – 35^0 C over night. The incubated tubes were observed for the number of survived nauplii for 24 hours. Graph was plotted for death percentage versus Log of concentration of the extract. This gives linear equation in the form of y = mx + c.

Calculation of LC_{50} value:

The extract concentrations were converted to the log. Death % was calculated as: (deaths/initial x 100).

Hence, from the graph using the equation of the straight line Lethal Concentration 50 (LC_{50}) value were calculated.

MTT Assay

One T-25 flask was trypisinsed and 5 ml of complete media was added to trypsinised HeLa cells. Centrifugation was done in a sterile 15 ml falcon tube at 500 rpm in the swinging bucked rotor (~400 x g) for 5 min. Media was removed and cells resuspended to 1.0 ml with complete media. Counting and recording cells per ml was done. Cells were diluted to 10,000 cells per ml using complete media. 100 µl of cells (10000 total cells) was added into each well and incubated overnight. Cells on Day two were treated with agonist, inhibitor or drug. Final volume was made 100 µl per well. 20 µl of 5 mg/ml MTT were added to each well. One set of wells with MTT but no cells were taken as control. Incubation was done for 3.5 hours at 37^oC in culture hood. Media was removed carefully. 150 µl MTT solvent (Isopropanol) was added. Covering with tinfoil and agitation of cells on orbital shaker was done for 15 min. Absorbance at 590 nm was taken with a reference filter of 620 nm.

The percentage growth inhibition was calculated using following formula,

%Cell inhibition= 100-{(At-Ab)/(Ac-Ab)}x100

where, At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

RESULTS AND DICUSSION

The bioprospecting of these medicinal herbs found in high altitude of Gosainkunda (4360 m) and Suryakunda (4800 m) region of Nepal, showed promising results from methanol extracts subjected to brine shrimp and antiproliferative assay. All the seven medicinal plants showed activity against brine shrimp (**Table 1**) and animal cell lines indicating the presence of secondary metabolites and medicinal properties. Correlation between Brine shrimp assay and MTT assay was observed as expected. The IC_{50} value obtained by MTT assay carried out and the measurement of an individual absorbance in 620 nm with specific calculation of death rate (**Table 2 and Table 3**). Thus, majority of plants showed a fairly good LC_{50} value with significant antiproliferative properties while some of them had noteworthy results. All the correlation between the brine shrimp activity and antiproliferative activities was found similar that supported their uses as medicine by the traditional healers and elderly peoples of our society.

For *Rhododendron setosum* , the LC_{50} value for 24 hours was observed as1862 ppm and whereas the same was observed as 0.0467 ppm for 48 hours. The very low LC_{50} value for 48 hours might be due to the natural death of brine shrimp upon exposure to the extract for very long period of time. The antiproliferative assay showed the IC_{50} value of 390 ppm suggesting the fair anticancerous property of the extract. Similarly *Eriophyton wallichii* showed LC_{50} value for 24 hours as 5.929 ppm and 0.028 ppm for 48 hours. The antiproliferative assay showed the IC_{50} value of lesser than 39 ppm indicating an excellent anticancerous properties. The least concentrated extract (0.039 mg/ml) of *Eriophyton wallichii* resulted the highest death (89.96%) rate of HeLa cells which matches its high use as wound healing properties of the in its traditional form.

For *Elsholtzia strobilifera*, the LC_{50} value for 24 hours was 186.2 ppm and that for 48 hours was 13.8 ppm. The antiproliferative assay showed the IC_{50} value of 78.10 ppm indicating the medium strength of the extract for anticancerous property. *Gentiana depressa* showed LC_{50} value for 24 hours as 177.8 ppm and that for 48 hours as 15.06 ppm. The antiproliferative assay showed IC_{50} value as 195.12 ppm indicating the strength of the extract as fairly good for anticancer properties.

Hedychium spicatum showed LC_{50} value for 24 hours as 100 ppm and 2.13 ppm for 48 hours whereas the antiproliferative assay for IC_{50} of the plant was observed as 48.77 ppm with very good anticancerous activity.

The LC_{50} value of *Rheum spp*. for 24 hours was observed as 1584.8 ppm and that for 48 hours was 25.1 ppm. The antiproliferative assay showed the IC_{50} value of 249.90 ppm indicating the extract as with fairly anticancerous properties. The root of Rhodiola *spp.* showed the LC_{50} value for 24 hours as 2398 ppm and that for 48 hours was 3.68 ppm. The antiproliferative assay showed the IC_{50} value of 101.47 ppm

Table 1. Table of Brine Shrimp Bioassay for cold methanol extracts (24 h)

Extract	Extract conc. (ppm) (A)	Log (A)	Initial nauplii	Nauplii survival n=1	Nauplii survival n=2	Nauplii survival n=3	Mean survival	Death	% death
Rheum spp.	10	1	10	10	10	10	10	0	0
	100	2	10	10	9	10	9.6	0.4	4
	1000	3	10	6	5	4	5	5	50
E. wallichi	10	1	10	7	4	8	4.75	5.25	52.5
	100	2	10	3	2	3	2.6	7.4	74
	1000	3	10	2	1	1	1.3	8.7	87
E. strobilifera	10	1	10	10	9	10	9.66	3.4	3.4
	100	2	10	10	8	10	9.33	0.67	6.7
	1000	3	10	0	0	0	0	10	100
G. depressa	10	1	10	10	9	10	9.66	3.4	3.4
	100	2	10	9	10	8	9	1	10
	1000	3	10	0	0	0	0	10	100
H. spicatum	10	1	10	10	10	10	10	0	0
	100	2	10	6	7	5	5	5	50
	1000	3	10	0	0	0	0	10	100
R. setosum	10	1	10	10	10	10	10	0	0
	100	2	10	9	10	10	9.66	0.34	3.4
	1000	3	10	5	7	8	6.66	3.34	33.4
Rhodiola spp. (root)	10	1	10	10	10	9	9.66	0.34	3.4
	100	2	10	10	9	8	9	1	10
	1000	3	10	5	6	5	5.33	4.67	46.7

Table 2. Table of absorbance

Ab= blank Absorb.	*Conc of extract (mg/ml)*	*R. setosum Absorb.*	*Rheum spp Absorb.*	*E. wallichi Absorb.*	*E. strobilifera Absorb.*	*Rhodiola spp(leaf) Absorb.*	*Rhodiola spp(roo) Absorb.*	*Hedychium spicatum Absorb.*	*G. depressa Absorb.*	*Ab of Egcg*	*Ac=Absorbance of control*
0.108	5	0.594	0.675	0.113	0.312	0.478	0.432	0.298	0.456	0.013	1.523
0.106	2.5	0.631	0.677	0.132	0.313	0.503	0.529	0.356	0.498	0.008	1.567
0.106	1.25	0.699	0.732	0.138	0.54	0.673	0.609	0.398	0.584	0.012	1.57
0.107	0.625	0.734	0.783	0.154	0.543	0.683	0.703	0.465	0.676	0.01	1.572
0.107	0.3125	0.873	0.791	0.167	0.713	0.751	0.758	0.586	0.762	0.006	2
0.105	0.156	1.02	0.91	0.198	0.783	0.942	0.793	0.658	0.858	0.008	1.582
0.106	0.0781	1.34	0.931	0.231	0.832	0.951	0.843	0.784	0.874	0.011	1.573
0.109	0.039	1.32	0.956	0.254	0.856	1.09	0.934	0.856	0.978	0.013	1.561
Avg = 0.1067											Avg = 1.561

Table 3. Death % of HeLa cells in various concentrations of extracts

Conc of extract (mg/ml)	*R. setosum Death %*	*Rheum spp Death %*	*E. wallichi Death %*	*E. strobilifera Death %*	*Rhodiola spp (leaf) Death %*	*Rhodiola spp (root) Death %*	*Hedychium spicatum Death %*	*G. depressa Death %*
5	66.46	60.9	99.65	85.85	74.43	77.59	86.84	75.94
2.5	63.91	60.76	98.35	85.78	72.71	70.92	82.81	73.05
1.25	59.24	56.98	97.93	70.18	61.03	65.42	79.93	67.14
0.625	56.83	53.48	96.83	69.98	60.34	55.18	75.32	60.08
0.3125	47.28	52.93	95.94	58.29	55.67	58.96	67.01	54.91
0.156	37.18	44.75	93.81	53.47	42.54	52.78	62.06	48.31
0.0781	15.18	43.3	91.54	50.1	41.92	49.34	53.4	47.21
0.039	16.56	41.59	81.96	48.45	32.37	43.09	49.45	40.06

which suggest that the extract has good anticancerous properties.

Hence, in comparing the seven different plants that was locally accepted as highly useful plants for different disease purpose, *Eriophyton wallichi* (5.929 ppm) and *Hedychium spicatum* (100 ppm) showed best cytotoxicity property during Brine shrimp bioassay (24 h). Antiproliferative effect was best observed in *E. wallichi* (less than 39 ppm*)* and *Hedychium spicatum* with 48.77 ppm. IC_{50} value of lesser than 39 ppm reason suggests that even the least concentrated extract 0.039 mg/ml resulted 89.96% death of HeLa cells indicating its excellent anticancerous-properties.

CONCLUSION

It was suggested by McLaughlin (1991) that brine shrimp (*Artemia salina*) lethality can be a predictor of potential antineoplastic activity. The brine shrimp lethality assay, then, represents a simple and inexpensive alternative screening technique to identify lead compounds with "cytotoxic" activity. In our project, we carry out brine shrimp lethality tests using a modification (Setzer, Talley, and Jackes, 1998) of the procedure described by McLaughlin (1991).Brine shrimp bioassay is mostly used as a pre-screen for plant extract because they provide a quick, inexpensive, and desirable alternative to testing on larger animals. It is known that a positive correlation exists between brine shrimp lethality and 9 KB (human nasopharyngeal carcinoma) cyto-toxicity; brine shrimp are therefore used in many prescreens for potential antitumor activity. As revealed in our results, extracts having lesser LC_{50} value in Brine shrimp Bioassay have corresponding lesser IC_{50} values.

The promising results obtained from the research of course stimulates further research on these plants, their safety concerns in human health and their preservation alongside conservations from the concerned authority.

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