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In vitro cytotoxicity of *Actephilla excelsa* and *Chrozophra parvifolia* different human cancer cell lines

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ABSTRACT

The Sulphorhodamine blue (SRB) assay and MTT assay was used for evaluating in vitro cytotoxic potential of two medicinal plants namely *Actephilla excelsa* and *Chrozophora parvifolia* and bitter gourd against five human cancer cell lines via petroleum ether, ethyl acetate, and methanol extracts. Results demonstrated that methanolic extract from *Actephilla excelsa* and methanolic extract from *Chrozophora parvifolia* possess selective anti-proliferative effect on human cancer cells taken from different tissues. However, methanolic extract of *Actephilla excelsa* was most effective against lung, liver and oral cancerous cells. *Chrpzophora parvifolia* (methanolic extract) inhibited the growth of central nervous system, lung and colon cancer cells whereas metahnolic extract of bitter gourd conquered the growth of 72% infected cells. *Actephilla excelsa* and *Chrozophora parvifolia* extracts are suggested to be subject of further studies to substantiate their role in suppression of cancerous cells in different parts of human body.

Keywords: *Actephilla excelsa, Chrozophora parvifolia*, SRB assay, MTT assay, Cytotoxicity.

1. INTRODUCTION

Cancer is the third leading cause of death worldwide, only preceded by cardiovascular disease, infectious and parasitic disease [1]. Interestingly, cancer has been the leading cause of death in Thailand for several years, with an increase in the death rate every year [2]. The plant kingdom has always been the favorite source of medication in all healing traditions all over the world and the use of plants as medicine is as old as human civilization. In India a number of traditional systems of medicine, *viz*., Ayurveda, Siddha, Unani *etc*. have been developed and are being utilized in the health care system of the country [3] From the earliest times, herbs have been prized for their pain-relieving and healing abilities and today we still rely largely on the curative properties of plants. According to World Health Organization, 80 % of the people living in rural areas depend on medicinal herbs as primary healthcare system. The synthetic anticancer remedies are beyond the reach of common man because of cost factor. Herbal medicines have a vital role in the prevention and treatment of cancer and medicinal herbs are commonly available and comparatively economical [4] *Actephila excelsa* (Dalz) Muller. Arg (Euphorbiaceac) is a shrub. It grows in temperate and tropical areas in the world. It is distributed in western peninsular india, ascending to an altitude of about 1200 m on rocky limestone hills, China, Bangladesh, Myanmar, Indonesia and Malaysia. In India it is commonly known as 'Lambonan' [5]. Leaves are obeveate or elliptic oblong, characterous petioles and leaf blades oblong lanceolate. Flowers are pinkish brown. Fruits arewoody capsule subglobose 3 lobed. Leaves of the plant were used traditionally as internal digestive disorders of G.I tract, respiratory tract system disorders, heart-blood circulatory system disorders, urinary tract system disorders and skin disorders. The two novel epimeric aromatic terpenoids were reported from leaf and stems of *Actephila excelsa* [6]. Macroscopic characters of the plant *Chrozopora parvifolia* was a prostrate are accending annual herb upto 50cm whitish or grayish stellate-bupeseent some times some what scabrid petioles 0.5-2(-4)cm long. Leaf blades triangular-ovate or ovate, some time shallowly trilovite, 1-4 (-5.5) into 0.7-3(-5)cm obtuse or rounded at apex [7].

2. MATERIALS AND METHODS

2.1. Collection and preparation of plant material

The fresh, healthy and disease free plants of *Actephilla excelsa* and *Chrozophra parvifolia* was collected from the natural habitats of

tirunelveli district, tamilnadu, india, in the month of june, 2012, and authenticated by Dr. V. Chelladurai (Retired Research Officer), Botany Govt. of India, tamilnadu, india. The samples were washed thoroughly in running tap water to remove soil particles and adhered debris and finally with sterile distilled water. The whole plants were shade dried and ground into fine powder. A voucher specimen (EM 600-2012-2013) has been preserved in the department of pharmacy, annamalainagar, annamalai university. The samples were stored in air tight container for further use.

2.2. Preparation of plant extract

A portion of shade dried aerial parts about 120 g of *Actephilla excelsa* and *Chrozophra parvifolia* was placed in a soxhlet apparatus. The extraction was performed with 800mL of methanol for 48 hrs at a temperature not exceeding the boiling point of the solvent. Extract was filtered through a 45µm filter. The Resulting extract was concentrated in a vacuum to dryness in a rotary evaporator to give the methanolic extract of *Actephilla excelsa* (6.8g) and the methanolic extract of *Chrozophra parvifolia* (7.2g). The extract was stored in a refrigerator at 4℃ for further use

2.3. Preliminary phytochemical analysis of methanolic extract of leaves of *Actephilla excelsa and Chrozophora parvifolia:*

2.3.1. Test for carbohydrates [8]

2.3.1.1. Molisch test

In this a small amount of leaves of *Cassia fistula* linn extract is treated with α-naphthol and concentrated sulphuric acid along the sides of the test tube. Purple colour or reddish violet colour at the junction between two liquids was formed. It indicates presence of carbohydrates.

2.3.1.2. Fehling's test

In this small amount of test extract is treated with equal quantity of Fehling's solution A and B is and Heat gently, brick red precipitate was formed. It indicates presence of carbohydrates.

2.3.1.3. Benedict's test

To the 5 mL of Benedict's reagent, added 8 drops of extraction solution. Mixed well, boiling the mixture vigorously for two minutes and then cool. Red precipitate was formed. It indicates presence of carbohydrates.

2.3.1.4. Barfoed's test

To the 5 mL of the Barfoed's solution added 0.5 ml of extraction solution and mixed well and heated to boiling, red precipitate was formed. It indicates presence of carbohydrates.

2.3.2. Test for alkaloids

About one gram of the powdered sample was extracted with 10 mL of 10% hydrochloric acid by boiling for five minutes on a water bath. The extract was filtered and the pH of the filtrate was adjusted to about 6 by adding a few drops of dilute ammonia solution and tested with litmus paper after which few drops of Dragendorff's, Mayer's and Wagner's reagent were added separately to aliquots of the filtrate in the test tubes. A reddish brown cream and reddish brown precipitate respectively indicates a positive test.

2.3.3. Test for steroids and sterols [9]

2.3.3.1. Libermann Burchard test

Leaves of *Cassia fistula linn* is dissolved in 2 ml of chloroform in a dry test tube. Then added10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. The solution becomes red, then later it was not changed to blue and bluish green colour, it indicates absence of steroids and sterols.

2.3.3.2. Salkowski test

Leaves of *Cassia fistula linn* was dissolved in chloroform and adds equal volume of conc. sulphuric acid. Bluish red cherry red and purple color is not formed in chloroform layer, and also green fluorescence was not formed, it's indicates the absence of steroids and sterols.

2.3.4. Test for glycosides [10]

2.3.4.1. Legal's test

The extract Sample is dissolved in pyridine sodium nitropruside solution is added to it and made alkaline. Pink red colour is produced. It's Indicates the presence of glycosides.

2.3.4.2. Baljet test

To the extract sample, sodium picrate solution is added. Yellow to orange colour is produced. It's indicates the presence of glycosides.

2.3.4.3. Borntrager test

Added a few mL of dilute sulphuric acid to the test solution. Boiled, filtered and extract the filtrate with ether or chloroform. Then organic layer is separated to which ammonia is added, pink, red or violet colour is produced in organic layer. It's indicates the presence of glycosides.

2.3.4.3. Keller Killiani test

Sample is dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of liquid reddish brown color is produced which gradually becomes blue. It's indicates the presence of glycosides.

2.3.5. Test for saponins

About one gram of the powdered sample was boiled with 10 mL of distilled water for ten minutes. The samples were filtered while hot, cooled and the following tests were carried out.

2.3.5.1. Frothing

2.5 mL of the filtrate was diluted to 10mLs with water and was shaken vigorously for 2 minutes. Frothing observed indicates a positive test.

2.3.5.2. Emulsification

2.5 mL of the filtrate was shaken vigorously for 2 minutes with a few drops of olive oil. An emulsified layer indicates a positive test.

2.3.6. Test for flavanoids [8]

A small quantity of the extract was dissolved in dilute sodium hydroxide and hydrochloric acid was added to the mixture. A yellow solution that turns colourless on addition of hydrochloric acid indicates the presence of flavonoids.

2.3.7. Test for triterpenoid [8]

In the test tube, 2 or 3 granules of tin was added, and dissolved in a 2mL of thionyl chloride solution and test solution was added. Pink colour is produced which indicates the presence of triterpenoid.

2.3.8. Test for protein and amino acid [10]

2.3.8.1. Biuret test

Added 1 ml of 40% sodium hydroxide and 2 drops of 1% copper sulphate to the extracts, a violet colour formed indicates the presence of proteins.

2.3.8.2. Ninhydrin test

Added 2 drops of freshly prepared 0.2% ninhydrin reagent to the extracts and heated. A blue colour developed indicating the presence of proteins, peptides or amino acids.

2.3.8.3. Xanthoprotein test

To the extracts, added 20% of sodium hydroxide. Orange colour was formed indicates presence of aromatic amino acid

2.4. Preparation of stock and working solutions

Stock solutions of 20mg/mL were prepared by dissolving ethanolic extract in dimethyl sulfoxide (DMSO), 50% ethanolic extract in 50% DMSO and aqueous extract in sterile water. Stock solutions were prepared atleast one day in advance and were not filtered, but the microbial contamination was controlled by addition of 1% gentamycin in complete growth medium *i.e.* used for dilution of stock solutions to make working test solutions of 200µg/mL.

2.5. Preparation of positive controls

Adriamycin, Mitomycin-C, 5-Flurouracil were prepared in distilled water while Paclitaxel was prepared in DMSO. Positive controls were then diluted in gentamycin medium to obtain the concentration of 2 X 10-3M.

2.6. Human cell lines

Human cancer cell lines namely of lung (NCI-H226), liver (hep-2) colon (HCT15) and Pancreatic (PACA-2) were grown in RPMI-1640 with 2 mM L-glutamine medium pH 7.2. Penicillin was dissolved in PBS and sterilized by filtering through 0.2µ filter in laminar air flow hood. The media was stored in refrigerator (2-8oC). Complete growth medium contains 10 % FCS. The medium for cryopreservation contains 20 % FCS and 10 % DMSO in growth medium. The cell lines were maintained at 370C in a 5% CO2 atmosphere with 95% humidity.

2.7. *In vitro* assay for cytotoxic activity

The anticancer activity was determined by the cytotoxic potential of the test material using human cancer cell lines which were allowed to grow on tissue culture plates in the presence of test material. The cell growth was measured using ELISA reader after staining with Sulforhodamine B dye (SRB) which binds to basic amino acid residues in the trichloroacetic acid (TCA) fixed cells

2.8. Preparation of Cell suspension for assay

Human cancer cell lines were grown in multiple tri conical flasks (TCFs) at 37℃ in an atmosphere of 5% CO2 and 90% relative humidity in complete growth medium to obtain enough number of cells. The flasks with cells at subconfluent stage were selected. Cells were harvested by treatment with Trypsin-EDTA. Cells were separated to single cell suspension by gentle pipetting action and the viable cells were counted in a hemocytometer using trypan blue. Cell viability at this stage should be >97%. Viable cell density was adjusted to 5,000 - 40,000 cells/100µl depending upon the cell line (Monks 1991). 100µl of cell suspension together with 100µl of complete growth medium was added into each well. The plates were incubated at 370C for 24 hours in an atmosphere of 5% CO2 and 90% relative humidity in a CO2 incubator. After 24 hours, the test material, DMSO (vehicle control) and positive control were added.

2.9. MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48h of incubation, 15µL of MTT (5mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula and the IC 50 was calculated using GraphPad Prism software (Table 3).

% Cell Inhibition = 100 - Abs (sample)/Abs (control) x100

2.10. Sulforhodamine B (SRB) assay

The antiproliferative SRB assay was performed to assess growth inhibition. This is a colorimetric assay which estimates cell number indirectly by staining total cellular protein with the SRB dye (Skehan 1990). The microtiter plates were taken out after 48 hours incubation of the cells with test materials and gently layered with chilled 50% TCA in all the wells to produce a final concentration of 10%. The tissue culture plates were incubated at 4°C for one hour to fix the cells attached to the bottom of the wells. The supernatant was then discarded. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc. Plates were air dried and stored until further use. SRB solution was added to each well of the plates and incubated at room temperature for 30 minutes. The unbound SRB was removed quickly by washing the wells five times with 1 % acetic acid and then air dried. 100µl of Tris buffer (0.01 M, pH 10.4was added and shaken gently for 5 minutes on a mechanical shaker. Optical density was recorded on ELISA reader at 515 nm

2.11. Statistical analysis

The experimental data were expressed as mean ± SEM. The significance of difference among the various treated groups and control group were analyzed by means of one-way ANOVA. The level of significance was set at $p < 0.05$. IC50 (inhibitory concentration which caused 50% inhibition) were estimated using linear regression method of plots of the percent of cell viability against the concentration of the tested compounds using Microsoft excel.

3. RESULTS AND DISCUSSION

3.1. Preliminary phytochemical screening

The methanolic extract of *Actephilla excelsa* was tested for alkaloid, carbohydrates, glycosides, phenols, tannins, flavonoids, terpenoids, phlobatannin, reducing sugar, volatile oil, were present and also steroid, saponin protein, aminoacids ,were absent (Table 1).

Table - 1: Shows preliminary qualitative chemical tests of *Actephilla excelsa*

Phytochemical constituents	*Actephilla excelsa*
Carbohydrates	+ve
Alkaloids	+ve
Steroids & sterols	-ve
Glycosides	+ve
Saponins	-ve
Flavonoids	+ve
Tannins & phenolic compound	+ve
Proteins & amino acids	-ve
Terpinoids	+ve
Reducing sugar	+ve
Phlobatannins	-ve

Table - 2: Shows preliminary qualitative chemical tests of *Chrozophora parvifolia*

Phytochemical constituents	*Chrozophora parvifolia*
Carbohydrates	+ve
Alkaloids	+ve
Steroids & sterols	-ve
Glycosides	+ve
Saponins	-ve
Flavonoids	+ve
Tannins & phenolic compound	+ve
Proteins & amino acids	-ve
Terpinoids	+ve
Reducing sugar	-ve
Phlobatannins	-ve

The methanolic extract of *Chrozophora parvifolia* was tested for alkaloid, phenols, tannins, flavonoids, terpenoids, phlobatannin, reducing sugar, volatile oil, were present and also steroid,

saponin, protein, aminoacids were absent (Table 2).

3.2. In vitro anticancer activity

The extracts and fractions were tested *in vitro* for its potential human cancer cell growth inhibitory effect on HeLa, HEp2 and A431 cancer cell lines using both MTT assay and SRB assay, a non radioactive, fast and economical assay wisely used to quantify cell viability and proliferation.

The IC 50 values of the extracts and fraction for the cell lines HeLa, HEp2 and A431, determined by both MTT assay and SRB assay was summarised and presented in Table 3. The cell line A431 was more sensitive among the cell line used and demonstrated low IC 50 values when compared to others. The Leaves extract of *Actephilla excelsa* and *Chrozophora parvifolia* were more active against the entire three cell lines tested and showed the IC 50 values of 26.8 to 32.9 µg/mL for A431, 43.7 to 80.7 µg/ml for HEp2 and 38.8 to 63.9 µg/ml for HeLa. Among the bark fractions, the M-CP showed higher cytotoxicity to all three cell lines and the IC 50 value was 38.8, 43.7 and 26.8 µg/mL for the cell lines HeLa, HEp2, and A431 respectively. However M-CP was not comparable with standard quercetin which dsplayed 32.2, 36.4 and 15.2 µg/ml for the cell lines HeLa, HEp2 and A431 respectively. Among the leaves extract of methanolic extract of *Actephilla excelsa* showed highest cytotoxicity but it was 2-3 fold higher IC 50 value when compared tomethanolic extract of *Chrozophora parcifolia*.

This is the first report to show the cytotoxic effect of the various extract of *chorozophora parvifoila*, particularly the metahnolic extract. However the cytotoxic effect of the leaves of methanolic extract of *Actephilla excelsa* and *Chorozophora parvifolia* may be due to the presence of high content of polyphenols particularly flavonoids.

Table - 3: IC 50 values of extracts and fractions for human cancer cell lines HeLa, HEp2 and A431 determined by MTT and SRB assay

Sample	HeLa			HEp2			A431		
	MTT IC 50 (µg/mL)	SRB IC 50 (µg/ml)	Average IC 50 (µg/ml)	MTT IC 50 (µg/ml)	SRB IC 50 (µg/ml)	Average IC 50 (µg/ml)	MTT IC 50 (µg/ml)	SRB IC 50 (µg/ml)	Average IC 50 (µg/ml)
STD	33.6	30.7	32.2	39.5	33.3	36.4	17.6	12.8	15.2
PE-AE	101.4	95.3	98.4	130.9	126.3	128.6	86.6	82.2	84.4
EA-AE	89.3	87.5	88.4	109.4	111.5	110.5	89.1	78.0	83.6
PE-CP	85.6	82.4	84.0	102.4	98.2	100.3	72.3	69.3	70.8
EA-CP	65.7	64.2	63.9	82.7	78.7	80.7	34.7	31.1	32.9
M-AE	57.9	55.7	56.8	72.1	67.9	70.0	35.4	32.3	33.8
M-CP	40.5	37.1	38.8	45.3	42.0	43.7	27.2	26.4	26.8

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