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Research Article

IN VITRO CYTOTOXICITY OF CARALLUMA SPECIES BY MTT AND TRYPAN BLUE DYE EXCLUSION

PAVAN KUMAR BELLAMAKONDI*[1], ASHOK GODAVARTHI [1], MOHAMMED IBRAHIM[2], SEETARAM KULKARNI[1], RAMACHANDRA NAIK.M[3], MARADAM SUNITHA[1]

[1]Radiant Research Services Pvt Ltd, Bangalore, India, [2] Nizam Institute of Pharmacy, Nalgonda, India, [3]Depart of PG studies and Research in Applied Botany, Kuvempu University, Shankaraghatta, India. Email: bpavan16@gmail.com

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ABSTRACT

Caralluma species have been reported for various traditional claims; the scientific data for possible activities have not yet been studied thoroughly. However the toxicity of a plant has to be studied for further exploration of various biological activities. Extracts were studied for their toxicity by MTT and trypan blue dye exclusion models against a panel of cancer, normal origin cell lines and EAC cells. The extracts found to be moderately toxic and showed dose dependent response.

Keywords: Caralluma, cytotoxicity, *invitro*, MTT and trypan blue.

INTRODUCTION

The use of medicinal plants for therapeutic use is known since time innumerable, continuous efforts are being made towards its improvement. Despite of its growing demand, there are still concerns associated with their safety [1]. Since plants produce secondary metabolites under different stressed conditions, these secondary metabolites apart from having therapeutic effect they also have to believe toxic compounds [2]. Therefore toxicity testing of phytochemicals and plant based extracts is of medicinal importance and must be considered in phytotherapy and other uses. Toxicity testing can reveal some of the risks that may be associated with their usage. This intern will facilitate in the identification of toxicants at an early stage of drug discovery and development from plant sources. Among the various studied models for toxicity study, cytotoxicity assays are one among the indispensable tools to predict toxicity [3]. Caralluma species have been used as a traditional medicinal plant all over the world for various therapeutic purposes [4-5]. Hence the present study was taken up with intent to understand the cytotoxic nature of extracts from Caralluma species, viz., *Caralluma lasiantha* (Wight) N.E.Br, *Caralluma* attenuate Wight, *Caralluma umbellata* Haw and *Caralluma diffusa* (Wight) N.E.Br which will help in dosage fixation for further exploration of their therapeutic efficacy.

MATERIALS AND METHODS

Media and Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Trypan blue, foetal bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640) and trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, glucose and antibiotics from Hi-Media Laboratories Ltd Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from Merck Ltd, Mumbai, India.

Plant collection and Extraction

The Plant materials were collected from different locations of southern India during the month april 2011. *Caralluma lasiantha* (Wight) N.E.Br (Gooty hills, Andhra Pradesh India), *Caralluma* attenuate Wight, *Caralluma umbellata* Haw. (Tirupathi, Andhra Pradesh. India) and *Caralluma diffusa* (Wight) N.E.Br. (Chitradurga, Karnataka. India). The plant materials were authenticated by Dr. Madava Chetty, S.V University, Tirupathi and were confirmed by comparing with the housed authenticated specimens. The collected plant materials were shade dried and powdered sample was extracted with methanol (M), water (A) successively and another set of material was extracted with methanol: water (60:40) (H) with soxhlet apparatus, the extracted materials were dried under reduced pressure.

Preparation of test sample

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with growth media supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Cytotoxicity by MTT

Preparation and Standardization of Stock cultures

Rat skeletal muscle *cell line (L6), Rat liver cell line (BRL3A),* African green monkey kidney cell line (Vero), Human breast cancer cell line (MCF 7), Human lung adenocarcinoma epithelial *cell line (A549),* Human colon adenocarcinoma *cell line (HT29),* Human cervix adenocarcinoma cell line (HeLa), Human prostate cancer cell line (PC 3), Human colon cancer *cell* line (Caco2) were procured from National Centre for Cell Sciences (NCCS) Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

PROCEDURE

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval.

After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT was added to each well later incubated for 3 h at 37° C in 5% CO_2 atmosphere. The formed formazan was solubilized with propanol. The absorbance was measured using a microplate reader (Biotek, USA) at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula, % Inhibition = 100 - (Test OD/untreated OD) × 100) and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves in the graph pad prism version 5.0 for each cell line [6].

Short term cytotoxicity study against EAC cells

Preparation and Standardization of Stock cultures

Cells were aspirated from the peritoneal cavity of tumour bearing mice and washed in PBS twice and counted using a haemocytometer. The cell population was adjusted to 2 X 10^5 cells/ml, with media.

PROCEDURE

To each containing 2 X 10^5 cells/ml, was incubated with different concentrations of methanolic extracts from four species to get final concentration from 62.5 to 1000 µg/ml. Cells were incubated for 3 hours at 37°C. After incubation, the cell death was evaluated using Trypan Blue exclusion method. To the cell suspension, 3 drops of Trypan Blue (0.5 % in PBS) was added and the cells were loaded immediately on to a haemocytometer. The number of Dead cells was counted and the percentage of dead cells was calculated. The percentage growth inhibition was calculated using the following formula, % Inhibition = 100 - (Total cells-dead cells/Total cells) × 100) and CTC_{50} value is generated from the dose-response curve in the graph pad prism version 5.0 [7].

Statistical analysis

Data representative of three independent experiments with similar results were presented as mean ± SD.

RESULTS

In the present study, the cytotoxic effect of three extracts from Caralluma species was determined. All the extracts were tested against a panel of normal and cancer cell lines at a range of 62.5 to 1000 µg/ml using MTT and trypan blue exclusion methods. The CTC_{50} values were shown separately for normal and cancer cell lines as in table 1 and 2 for MTT assay; the CTC_{50} values for short term study are depicted in fig.1. The cytotoxicity of three different extracts viz., methanolic, aqueous and hydro methanolic from studied species showed similar pattern with respect to their specificity towards toxicity. The toxicity of extracts are in increasing order, methanolic > hydro methanolic > aqueous extract.

Among the studied normal cell lines, extracts exhibited high toxicity towards Vero cell line with CTC_{50} ranging from 368.70±3.4 to 930+2.3 µg/ml followed to BRL3A and L6 cell lines as shown in table 1. The extracts also exhibited moderate cytotoxicity against cancer cell lines, among the cancer cell lines, PC3 was found to have less toxic effect from extracts where as $Caco_2$ cell line showed higher affinity towards cytotoxicity as CTC_{50} was below to 1000 µg/ml. Overall the CTC_{50} was found to be in the range of 121.60±3.2 to >1000 µg/ml as shown in table 2.In short term study, only methanolic extracts from four species was evaluated, against EAC cells all methanolic extracts possessed cytotoxicity; the CTC_{50} was found at 191.3±0.92 to 291.8±3.17 µg/ml.

DISCUSSION

The cytotoxic effect of the crude extracts from *C.lasiantha, C.diffusa, C. umbellata* and *C. attenuata* were investigated using MTT and trypan blue exclusion methods. MTT assay is based on the reduction of yellow tetrazolium MTT to a purple formazan dye by mitochondrial succinate dehydrogenase [8]. Similarly, the trypan blue assay based on the assumption that the dead cells will take the dye and viable cells won't [7]. From the study, it was observed that extracts showed moderate cytotoxic against both cancer and normal cell lines. Toxicity from plants may be attributed to majorly from alkaloids, glycosides, saponins, polyacetylenes [9-10]; previous phytochemical reports from caralluma species suggest the presence of glycosides and saponins as key phytochemicals [10-12]. Hence the observed cytotoxicity from selected species may be from observed phytoconstituents, which may partially support our present finding. The cytotoxicity of extracts found to be in dose dependent and non selective as reflected by uniform CTC_{50} values independent of cell line origin. The species from Caralluma are reported to use as vegetables in Southern India [4] and also the species are known possess various biological activities [13-15], from the study it was observed that extracts are found to be moderately toxic. Hence, there is important need for further investigations on identification of phytoconstituents responsible for toxicity and also studying nature of toxicity using animal models.

CONCLUSION

The *invitro* cytotoxicity assays offers quick, simple and cost-efficient way of testing the toxicity and forms an important tool for high throughput screening of plant extracts. In addition, they have significant impact in the implementation of the three R's; the reduction of number of animals used, refinement of animal test models and replacement of animal in research. From the present findings, it can be concluded that the studied extracts shows moderate toxicity against both cells irrespective of their origin. Hence the extracts need to be thoroughly studied using animal models.

Table 1: Cytotoxicity of test extracts against Normal cell lines by MTT assay

Sl. No	Extract	Cell line (CTC_{50} µg/ml ± SD) Average of 3 replicates		
		L6	BRL3A	Vero
1	MCL	>1000.00	757.03±10.4	495.20±5.4
2	ACL	>1000.00	756.00±10.0	886.67±11.5
3	HCL	>1000.00	604.20±9.9	669.07±10.9
4	MCD	760.00±14.1	664.50±10.3	368.70±3.4
5	ACD	>1000.00	818.23±2.3	713.57±9.8
6	HCD	>1000.00	327.20±2.1	513.57±6.0
7	MCU	220.10±11.1	556.50±10.5	738.57±1.8
8	ACU	549.53±8.8	688.07±3.8	733.23±7.4
9	HCU	687.90±16.7	337.00±14.4	930.00+2.3
10	MCA	>1000.00	401.70±8.5	476.50±0.9
11	ACA	>1000.00	631.23±13.0	900.00±1.1
12	HCA	>1000.00	351.60±14.0	632.90±2.7

(CL) ***C.lasiantha*****, (CA)** ***C.*****attenuta, (CU)** ***C. umbellata*** **and (CD)** ***C. diffusa***

Table 2: Cytotoxicity of test extracts against Cancer cell lines by MTT assay.

Sl.No	Extract	Cell line (CTC_{50} µg/ml ± SD) Average of 3 replicates					
		MCF7	HT29	A549	HeLa	PC3	Caco2
1	MCL	289.30±12.6	961.90±11.4	125.70±13.5	393.87±9.5	>1000	391.30±3.8
2	ACL	>1000	974.60±8.0	>1000	>1000.00	>1000	720.40±13.5
3	HCL	611.70±16.0	973.30±5.3	223.73±8.8	984.70±9.6	>1000	515.90±0.7
4	MCD	415.73±13.3	959.17±7.4	121.60±3.2	351.10±1.0	>1000	348.70±4.3
5	ACD	>1000	>1000	>1000	>1000	>1000	963.33±11.5
6	HCD	289.83±11.7	>1000	260.83±2.1	477.30±3.8	>1000	160.93±13.8
7	MCU	333.40±8.2	385.13±13.5	150.73±9.6	170.33±8.0	>1000	285.73±1.5
8	ACU	>1000	>1000	>1000	>1000	>1000	416.90±3.4
9	HCU	>1000	964.03±12.8	279.13±2.0	>1000	>1000	989.50±8.8
10	MCA	127.60±6.0	344.83±1.5	638.23±14.4	141.27±0.5	>1000	217.73±1.2
11	ACA	293.87±10.0	640.50±5.9	>1000	>1000	>1000	709.30±13.1
12	HCA	437.10±16.6	500.33±6.7	>1000	514.30±13.3	610±10.00	454.43±2.5

(CL) ***C.lasiantha*****, (CA)** ***C.attenuta*****, (CU)** ***C. umbellata*** **and (CD)** ***C. diffusa***

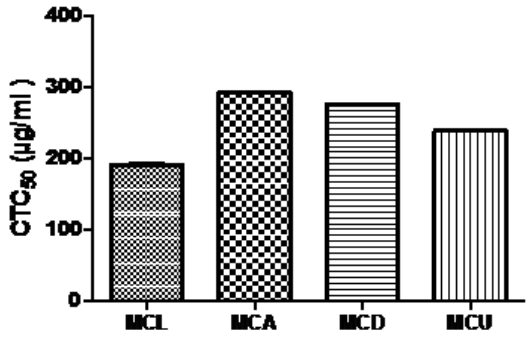

Fig.1: Short term cytotoxicity study against EAC cells by trypan blue assay. Values are CTC50 µg/ml ± SD, average of 3 replicates. Methanolic extracts of C.lasiantha (CL), C.attenuta (CA), C. umbellata (CU) and C. diffusa (CD).

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