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Screening for cytotoxic activity of *Habenaria longicorniculata* J graham tubers- an *in vitro* study

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ABSTRACT

About: *Habenaria longicorniculata* J. Graham are tuberous orchid, the tubers utilized by flok healers in cancer managemnet, as a rejuvenator. A study has been planned to evaluate *In-vitro* cytotoxicity of tuber extract against selected cell lines. **Materials and Methods:** *H. longicorniculata* J.Graham identified, uprooted during their flowering time. Tuber extract of this plant used for its *In-vitro* cytotoxicity against selected cell lines of Human Breast cancer (MCF 7), Human Liver carcinoma (HepG2), and Human cervix adenocarcinoma (HeLa) cells as per standard protocol. **Results:** Tuber Extract exhibited a CTC_{50} value of >1000 on MCF 7, HepG2 and HeLa cell lines. The results from the MTT assay indicate that 72hr extract incubation with the combined extracts is toxic to the cells and the level of damage is concentration dependent.

Keywords: *Habenaria longicorniculata*, *In-vitro* study, Cytotoxic activity, Human Breast cancer (MCF 7), Human Liver carcinoma (HepG2), Human cervix adenocarcinoma (HeLa).

INTRODUCTION

Changed life style, pollution, stress related factors are prime cause of increased incidence of malignant disorders globally [1]. Secondary metabolites of few plant species have proved toxic on these cancer cells, simultaneously not affecting other normal cells[2]. Many plants have been screened to test their efficacy in cancer since 1950 [3]. A great number of plant species have been investigated for cytotoxic, antitumour and anticancer activities which included both in-vitro and in-vivo models [4]. These preclinical studies have significant contribution in novel drug discovery in cancer treatment, incorporating a variety of cell lines and tumor types in the pre-screening, screening protocols of potential anticancer drugs [5]. As cancer was primarily considered a disease of uncontrolled cell division, by measuring the regression in tumor size, identification of a cytotoxic or an antiproliferative compound was considered as the main objective endpoint of efficacy of a compound in preclinical and clinical anticancer drug development for decades [6]. A potent anticancer drug must kill or weaken cancer cells without injuring normal cells [7]. Traditional/ verbal community has a vast knowledge on various anticancer drugs, but pharmacological action yet to be derived [8].

Habenaria longicorniculata J. Graham is an orchid, found at peninsular India belonging to family *Orchidaceae*, found commonly at hilly, slope areas among grasses [9]. Orchid usually gives white flowers in the month of August, during which underground tubers to be collected [10]. Traditional physicians use these tubers in the treatment of malignancy, as a rejuvenator also in many ailments [11]. Tubers said to be beneficial in wasting disease, fever, disorders of blood, haemorrhage and specifically used as overall rejuvenator [12]. The tubers are said to possess antioxidant property. Today whole world is looking for scientific evidences for traditional claims. Hence a study has been planned to evaluate *In-vitro* cytotoxicity of tuber extract against selected cell lines of Human Breast cancer (MCF 7), Human Liver carcinoma (HepG2), and Human cervix adenocarcinoma (HeLa) cells.

Cell culture technique an exclusive research in cellular and molecular biology, which provide exceptional study design in the field of normal physiology and biochemistry of cells (e.g., metabolic studies, aging)[13]. This is also used in new herb screening, drug preparation (e.g., vaccines, therapeutic proteins). Uniqueness in consistency and reproducibility of results using batch of clonal cells, make it popular among invitro study[14]. Thus based on above all mentioned factor a study has been designed to evaluate In-vitro cytotoxic activity of *Habenaria longicorniculata* J.Graham tubers on few malignant cell lines.

MATERIALS AND METHODS

Tubers of *Habenaria longicorniculata* J. Graham were collected during their flowering season, from their natural habitat. Plant sample cleaned using water, authenticated, roots detached from plant and shade dried.

Tuber extract taken for its *In-vitro* cytotoxicity against selected cell lines of Human Breast cancer (MCF 7), Human Liver carcinoma (HepG2), and Human cervix adenocarcinoma (HeLa) cells [15].

METHODOLOGY

Test substance was prepared at concentrations ranging from 1000 µg/ml to7.8 µg/ml to determine the percentage growth inhibition of the test substance on MCF 7, HepG2 and HeLa cell lines [16].

Preparation of test solution

For cytotoxicity studies, 10mg of test drug was separately weighed and volume was made up with DMEM-HG/MEM supplemented with 2% inactivated FBS to obtain a stock solution of 10% w/v concentration and sterilized by 0.22µ syringe filtration. Serial two-fold dilution was arranged from this for carrying out cytotoxicity studies [17].

Cell lines and Culture medium

Human breast cancer (MCF 7), Human Liver carcinoma (HepG2), and Human cervix adenocarcinoma (HeLa) cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM-HG/MEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a 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 well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India) [18].

Cytotoxicity Study

The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using DMEM-HG/MEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension was added. After 24 hours, when a partial monolayer was formed, the supernatant was skimmed off, the monolayer washed 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 72 hr in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24hr interval. After 72 hr, the drug solutions in the wells were thrown off and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 hr at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the Standard formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values was generated from the dose-response curves for each cell line [19].

RESULTS

Tuber extract of *Habenaria longicorniculata* J Graham showed results for MCF 7 Human breast carcinoma cells, HepG2 Human Liver carcinoma cells, HeLa Human Cervical adenocarcinoma cells as follows. The percentage growth inhibition was calculated using the Standard formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values was generated from the dose-response curves for each cell line.

1.Cytotoxic properties against MCF 7 cell line:

At the test concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.5 and 7.8, the Percentage of Cytotoxicity inhibition was 37.34, 32.87, 26.40, 18.75, 13.19, 11.70, 10.28 and 1.88 respectively. The percentage growth inhibition was calculated using the Standard formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values was generated from the dose-response curves for each cell line. (Table 1)

Table 1: Cytotoxic properties of *Habenaria longicorniculata* J Graham against MCF 7 cell line

Sl. No.	Test Compound	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	Tuber Extract	1000	37.34±0.38	>1000
		500	32.87±0.18	
		250	26.40±0.24	
		125	18.75±0.22	
		62.5	13.19±0.29	
		31.25	11.70±0.30	
		15.5	10.28±0.30	
		7.8	1.88±0.11	

2. Cytotoxic properties against HepG2 cell line

At the test concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.5 and 7.8, the Percentage of Cytotoxicity inhibition was 33.07, 24.75, 17.30, 13.59, 12.51, 07.45, 02.15 and 0.82 respectively. (Table 2)

Table 2: Cytotoxic properties of *Habenaria longicorniculata* J Graham against HepG2 cell line

Sl. No.	Name of Test Compound	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	Tuber Extract	1000	33.07±0.31	>1000
		500	24.75±0.28	
		250	17.30±0.20	
		125	13.59±0.36	
		62.5	12.51±0.52	
		31.25	7.45±0.31	
		15.5	2.15±0.27	
		7.8	0.82±0.46	

3. Cytotoxic properties against HeLa cell line

At the test concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.5 and 7.8, the Percentage of Cytotoxicity inhibition was 45.81, 41.74, 40.20, 38.64, 36.48, 35.09, 24.96 and 8.04 respectively. (Table 3)

Table 3: Cytotoxic properties of *Habenaria longicorniculata* J Graham against HeLa cell line

Sl. No.	Name of Test Compound	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	Tuber Extract	1000	45.81±0.68	>1000
		500	41.74±0.38	
		250	40.20±0.16	
		125	38.64±0.43	
		62.5	36.48±0.32	
		31.25	35.09±0.63	
		15.5	24.96±0.78	
		7.8	8.04±0.47	

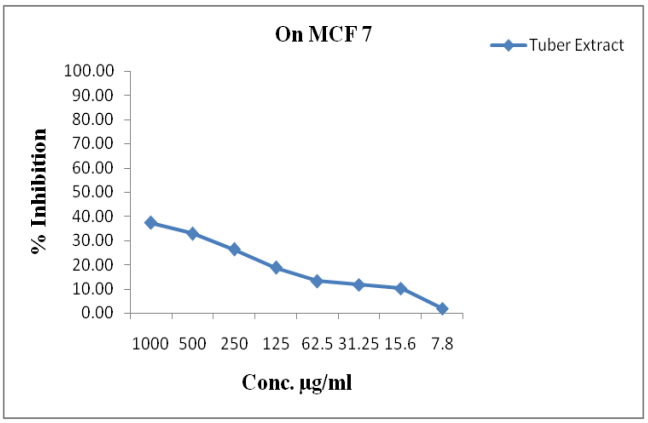

Figure 1: Graph of cytotoxic effect of test substance on MCF 7.

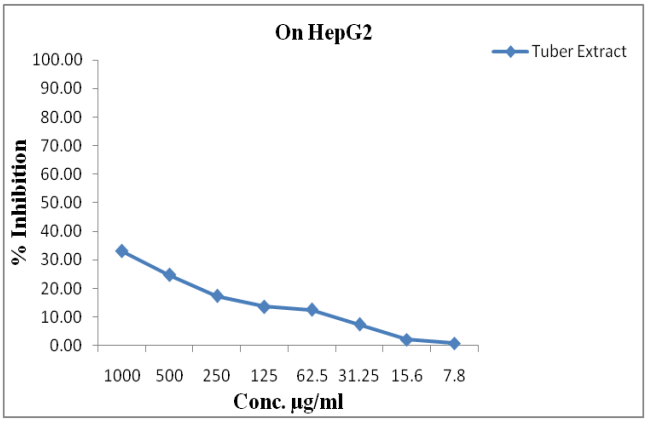

Figure 2: Graph of cytotoxic effect of test substance on HepG2

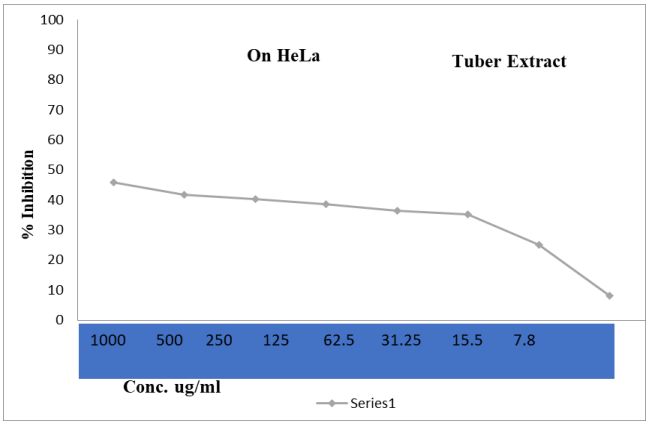

Figure 3: Graph of cytotoxic effect of test substance on HeLa cell line.

DISCUSSION

Herbs have been used since centuries as therapeutics to cure illness as well as to build up immunity. Plants have a major role in maintaining health of this earth as well all living beings. *In vitro* cytotoxicity screening models afford significant introductory data to help select plant extracts with probable anticancerous activity[20]. Although *Habenaria longicorniculata* J.Graham tubers have valuable pharmacological effects, the comprehensive awareness about its cytotoxic activity has been lacking.

In-vitro cytotoxicity of Tubers extract of *Habenaria longicorniculata* J Graham was tested against MCF 7, HepG2 and HeLa cell line. Test substance was taken at concentrations ranging from 1000 µg/ml to 7.8 µg/ml to determine the percentage growth inhibition of the test substance on MCF 7, HepG2 and HeLa cell lines. The test substance extract exhibited a CTC_{50} value of >1000 on MCF 7, HepG2 and HeLa cell lines.

The MTT assay is a delicate, measurable and consistent colorimetric assay that measure cell capability[21]. This is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue/purple formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cells lines. According to the American National Cancer Institute (NCI), the criteria of cytotoxicity for crude extracts is an $CC_{50} < 30$ µg/mL after an exposure time of 72 h in a preliminary assay [22]. Tuber Extract of *Habenaria longicorniculata* J Graham met these criteria with CC_{50} value less than 30 µg/mL with the crude extract being the most cytotoxic.

Test drug extract (HL) showed results for MCF 7 Human breast carcinoma cells at the test concentration of 1000 µg/ml - 37.34% and at 500 µg/ml - 32.87% of cytotoxicity inhibition was seen respectively. Whereas for HepG2 Human Liver carcinoma cells at the test concentration of 1000 µg/ml - 33.07% and at 500 µg/ml - 24.75% of cytotoxicity inhibition was observed. For HeLa Human Cervical adenocarcinoma cells at the test concentration of 1000 µg/ml - 45.81% and at 500 µg/ml - 41.74% of cytotoxicity inhibition was found by test drug (HL).

The results from the MTT assay indicate that 72hr extract incubation with the combined extracts is toxic to the cells and the level of damage is concentration dependent. Unwanted reactions can give rise to high background absorbance values especially in herbal extracts when antioxidants in the extracts directly react with MTT or formazan. This occurrence was minimal in these experiments because the blanks (media without cells) did not give background reactions. Test drug extract has shown considerable cytotoxic activity on malignant cells, thus proving its anticancer property.

CONCLUSION

Tuber Extract of *Habenaria longicorniculata* J Graham was tested for *In vitro* cytotoxicity studies against MCF 7 (Human breast cancer), Human Liver carcinoma (HepG2), and Human cervix adenocarcinoma (HeLa) cells by MTT assay exposing the cells to different concentrations of test substance. Tuber Extract (HL) exhibited a CTC_{50} value of >1000 on MCF 7, HepG2 and HeLa cell lines. The results from the MTT assay indicate that 72hr extract incubation with the combined extracts is toxic to the cells and the level of damage is concentration dependent. Thus, a promising anticancer drug, to confirm with further experimental models.

Conflict of interest

The authors declare no conflict of interests

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