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Research Article

IN VITRO TOXICITY STUDY OF AN AYURVEDIC FORMULATION "KRSHINADI CHURNA" ON HELA CELLS

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ABSTRACT

Krshnadi Churna is a classical ayurvedic formulation used for the treatment of infantile diarrhea with fiver, asthma in children and infantile vomiting etc. This formulation have *Piper Longum* (Krshna) (Fr), *Aconitum Hetrophyllum* (Aruna)(Rt), *Cyperus Rotumdus* (Musta)(Rz), *Pistacia Integerrima* (Gall). *Aconitum Hetrophyllum* is the most important ingredient of the formulation. But some literature reports reveled that few *Aconitum* species are extremely poisonous in nature. Hence it was necessary to evaluate its toxicity. We have selected *Krshnadi Churna* for *In-Vitro* toxicity study. The formula was referred from Ayurvedic Pharmacopoeia of India. The study clearly reveals the nontotoxic activity of formulation against Hela cell line with IC50 value of 40.25 µg/ml for hydroethanolic fraction respectively by MTT assay. Vincristine sulphate was used as standard cytotoxic agent and the IC50 of test samples are compared with standard. The IC50 value of vicristine sulphate is 47.56 µg/ml.

INTRODUCTION

There are around 3,000-5,000 years ago ayurvedic medicine originated. These formulation are taken from the ancient Vedic text books or Vedas (Books of Ayurveda, Siddha, Unani)the ancient religious and philosophical texts that are oldest surviving healing system. According to the texts, Ayurveda is the most ancient science of life having a holistic health approaches in Sanskrit Ayur means "Life" and Vedas means "Knowledge" so Ayurveda is defined as "The knowledge of living" or "The Science of Longevity".[1-2] There are medicines are present in traditional Ayurvedic text books such as Charaka Samhita, Sushruta Samhita etc. The manufacturing company follows the same formula and prepares medicines. For e.g bhasmas, asavas, arishtas, taila,Churna etc [3] Krshnadi Churna is a classical ayurvedic formulation used for the treatment of infantile diarrhea with fiver, asthma in children and infantile vomiting etc. This formulation have *Piper Longum* (Krshna) (Fr), *Aconitum Hetrophyllum* (Aruna)(Rt), *Cyperus Rotumdus* (Musta)(Rz), *Pistacia Integerrima* (Gall). *Aconitum Hetrophyllum* is the most important ingredient of the formulation. But some literature reports reveled that few *Aconitum* species are extremely poisonous in nature. Hence it was necessary to evaluate its toxicity. We have selected *Krshnadi Churna* for *In-Vitro* toxicity study. The formula was referred from Ayurvedic Pharmacopoeia of India. [4]

Benefits of Ayurvedic formulations

Now a day's Ayurvedic medicines is widely practiced in modern India and are becoming popular day by day throughout the world as compared with allopathic medicines because it has no side effects. Ayurvedic treatment is non-invasive and non-toxic, so it can be used safely as an alternative therapy or along-side conventional therapies. There are number of plants, herbs present in nature which are useful in classical formulations. According to the ancient Ayurvedic texts, the main goal of Ayurveda is prevention as well as promotion of the body's own healing capacity. Ayurveda mainly concentrates on the main cause of disease and cures it from root level. Ayurveda has been used to treat different disorders like acne, allergies, asthma, anxiety, arthritis, chronic fatigue syndrome, colds, colitis, constipation, depression, diabetes, flu, heart disease, hypertension, immune problems, inflammation, insomnia, nervous disorders, obesity, skin problems, and ulcers.[5],[6]

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In vitro Study

In vitro studies are conducted using components of an organism that have been isolated from their usual biological surroundings, such as microorganisms, cells, or biological molecules. For example, microrganisms or cells can be studied in artificial culture media, and proteins can be examined in solutions**.** [7]

Benefits of In-vitro methods

Advantages and disadvantages of in vitro and In-vivo methods for producing mAb are highlighted in this section. It should be noted that it is likely that in vitro methods will meet more than 90% of the needs for mAb. Some of the advantages and disadvantages are concerned with animal-welfare issues[8,]

HeLa Cells

Hela celline are most widely used in scientific research. It is an oldest and most commonly used human cell line. It is a human cervical cancer cells. The HeLa cell line was derived for the use of cancer research. These cells proliferate abnormally rapidly, even compared to other cancer cells.[9,10]

Table No. 1 HeLa Celline

Organism	Homo sapiens, human
Tissue	Cervix
Cell Type	Epithelial
Morphology	Epithelial
Culture Properties	Adherent

Formulation Profile

Krsnadi Churna

Krsihnadi Churna is the formulation which is given in a official book "The ayurvedic formulary of India" This is the formulation which are used for the treatment of infantile diarrhea with fiver, Asthma in Children, Infantile vomiting. This formulation contents plants Piper longum(Krshna), Aconitum hetrophyllum(Aruna)Rt, Cyperus Rotumdus (Musta)Rz, Pistacia Integerrima(Gall). [4]

Dose:-1-3 Gram Twice a day with Honey

Route of Administration – Oral route

Table No 2 Composition of Krsnadi Churna [3]

Ayurvedic Name	Scientific name	Quantity
Krsna	Piper longum (Fr)	1 part
Aruna	Aconitum hetrophyllum (Rt)	1 part
Mustaka	Cyperus rotundus (Rz)	1 part
Srngika	Pistacia integerrima (GI)	1 part

MATERIALS AND METHODS

Objective

The objective of the present study was to establish the safety of one of the most important classical ayurvedic formulation Krshnadi Churna by conducting its Toxicity study on Hella Cells.

Experimental Methods

A HeLa human cancer epithelial cell line was used. HeLa cell was procured from National Centre for Cell Sciences, Pune, India. Plant ingredients were collected of formulation according to the Ayurvedic formulary of India and identified by the macroscopic character. All the plant ingredients had dried at the sun light, and after that Plant Materials had being formulated in powdered form with the help of Pestle mortar, than mixed all the powder ingredients accordingly to the Ayurvedic formulary of India for the formulation of "Krsnadi Churna DMEM was prepared from powdered media (Himedia laboratories, Mumbai, India) with L- glutamine, without sodium bicarbonate ($NaHCO_3$). The powder provided in the vial was dissolved in 1000 ml of autoclaved double distilled water (DDW). At 900ml of double distilled water (DDW), contents of 1 unit vial were added at room temperature with constant stirring. Cultures grown in media supplemented with 10% Foetal Bovine Serum were viewed using an inverted phase contrast microscope to observe growth and morphology of the cells. When the pH of the medium turned acidic, indicating overgrowth of cells, the HeLa cells attained confluence, the media was removed by aspiration and the cells were washed with sterilized phosphate buffered saline. The cells were then trypsinised with 0.25% trypsin for 2-3 min and subsequently washed with DMEM media without FBS. The cells were mixed with fresh media and finally reseeded at a concentration of 1x106 cells/ml into fresh culture flasks containing DMEM media supplemented with 10% FBS. The flasks were tilted gently to ensure uniform distribution of cells which were then cultured in a CO_2 incubator in a humidified atmosphere with 5% CO_2 at 37^0C. The mono layer cells were detached and single cell suspensions were made using trypsin-ethylene diamine tetra acetic acid (EDTA). A hemocytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 5% FBS in order to obtain final density of 1x106 cells/ml.96-well plates

Cell lines

A HeLa human cancer epithelial cell line was used. HeLa cell was procured from National Centre for Cell Sciences, Pune, India. HeLa was maintained and sub cultured at Columbia Institute of Pharmacy Raipur.

Preparation of Ayurvedic formulation

Plant ingredients were collected of formulation according to the Ayurvedic formulary of India and identified by the macroscopic character. All the plant ingredients had dried at the sun light, and after that Plant Materials had being formulated in powdered form with the help of Pestle mortar, than mixed all the powder ingredients accordingly to the Ayurvedic formulary of India for the formulation of "Krsnadi Churna

Preparation of media

DMEM was prepared from powdered media (Himedia laboratories, Mumbai, India) with L- glutamine, without sodium bicarbonate (NaHCO3). The powder provided in the vial was dissolved in 1000 ml of autoclaved double distilled water (DDW). At 900ml of double distilled water (DDW), contents of 1 unit vial were added at room temperature with constant stirring. In this, 1500 mg of NaHCO3 ((Himedia laboratories, Mumbai, India) was added and stirred well. The pH of the solution was adjusted to a range of 7.1- 7.5 1N HCl and 1N NaOH. Final volume was made upto 1000ml with

DDW and sterilized by filtering through autoclaved cellulose acetate membrane filter with a pore size of 0.2 µm and diameter of 47 mm in a filter unit, using vacuum pump To the filtered media 2.5 ml of antibiotic solution (Himedia Laboratories, Mumbai, India) was added and stored in a refrigerator at 2-8°C until further use. At the time of use media was supplemented with 10% Foetal Bovine Serum (FBS) procured from Himedia Laboratories, Mumbai, India.

Subculture of Cell Lines

Cultures grown in media supplemented with 10% Foetal Bovine Serum were viewed using an inverted phase contrast microscope to observe growth and morphology of the cells. When the pH of the medium turned acidic, indicating overgrowth of cells, the HeLa cells attained confluence, the media was removed by aspiration and the cells were washed with sterilized phosphate buffered saline. The cells were then trypsinised with 0.25% trypsin for 2-3 min and subsequently washed with DMEM media without FBS. The cells were mixed with fresh media and finally reseeded at a concentration of 1x106 cells/ml into fresh culture flasks containing DMEM media supplemented with 10% FBS. The flasks were tilted gently to ensure uniform distribution of cells which were then cultured in a CO2 incubator in a humidified atmosphere with 5% CO2 at 37 ^{0}C [11,12]

Cell treatment

The mono layer cells were detached and single cell suspensions were made using trypsin-ethylene diamine tetra acetic acid (EDTA). A hemocytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 5% FBS in order to obtain final density of 1x106 cells/ml.96-well plates at plating density of 10,000 cells/well were seeded with one hundred microlitres per well of cell suspension and incubated for cell attachment at37° C,5% CO2, 95%airand100%relativehumidity. The cells were treated with serial concentrations of the test samples after 24 hr. Serial dilution method was used for preparing test samples of different concentrations. Cells were initially dissolved in dimethylsulfoxide (DMSO) and further diluted with serum free medium to obtain twice the desired final maximum test concentration. The required final drug concentrations of 10, 20, 40, 80, 100 µg/ml were obtained by adding aliquots of 100 µl of the different drug dilutions to the appropriate wells already containing 100 µl of medium. After addition of the drug the plates were incubated for an additional 48 hr at 37°C, 5% CO2, 95% air and 100% relative humidity. The medium without samples served as control and triplicate was maintained for all concentrations.

Micro culture tetrazolium (MTT) assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0x106 cells/ml using DMEM medium containing 10% fetal bovine serum. To each well 100 µl of the diluted cell suspension was added. After 24 hours, the Different concentration of test & Standard was added and final volume of the well was made up to 200µl with medium (Serum Free) to the cells. The plates was then incubated at 37^{0}C for optimum time, and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded and 20µl of MTT was added to each well. The plates shall be gently shaken and incubated for 3 hours at 37^{o}C. The supernatant has to be removed and 200µl of solubilization solution was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured at a wavelength of 570nm. The percentage growth inhibition was calculated.

RESULT AND DISCUSSION

Table no 4 MTT Assay Results

Concentration (µg/ml)	% Cell Viability	
	HAEAc	Standard (Vincristine)
10	89.39±0.021 [a]	66.4±0.033 [a]
20	84.45±0.046 [a]	54.51±0.021 [a]
40	83.95±0.032 [a]	41.45±0.030 [a]
80	71.27±0.016 [a]	12.64±0.046 [a]
100	70.58±0.041 [a]	4.41±0.022 [a]

Results shows a Significant difference from standard by two way ANNOVA followed by Bonferroni's multiple Comparison test, a= ****p<) 0001

HAECc- Hydroethanolic Ayurvedic formulation

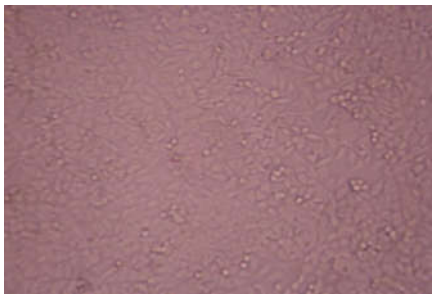

Fig 1 Control cells

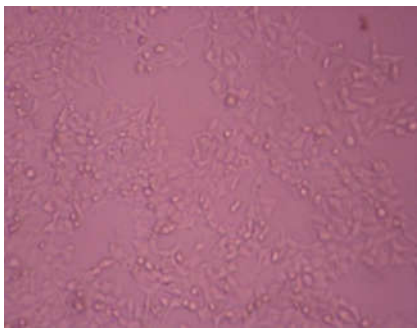

Fig 2 Formulation treated cells

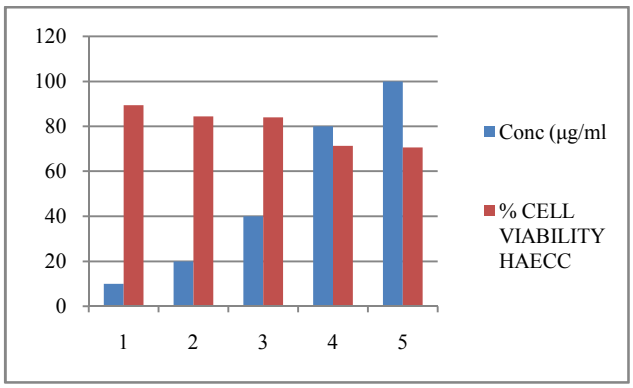

Fig 3 % cell viability after treating with HAECC

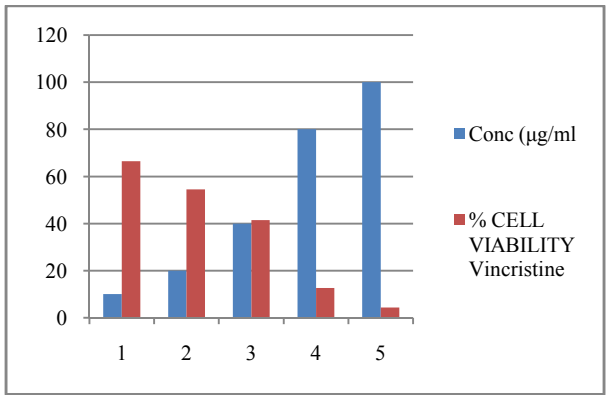

Fig 4 % cell viability after treating with Standard Vincristin Sulphate

In this in vitro toxicity assay, it is observed the formulation of Krshnadi Churna. Shows non-totoxic activity against Hela cell line with IC50 value of 40.25 µg/ml for hydroethanolic fraction respectively by MTT assay. Vincristine sulphate is used as standard cytotoxic agent and the IC50 of test samples are compared with standard. The IC50 value of vicristine sulphate is 47.56 µg/ml. Parag R. Patel et al[1] done Cytotoxic activity for Rubia Cardifolia for different fraction against Hela cells and reported the significant to good cytotoxic activity,because Cytotoxic drug have toxicity Thus in relation to that result Krshnadi Churna formulation is having non- totoxic activity.

CONCLUSION

On the basis of the above result it can be concluded that Hydro ethenolic Ayurvedic formulation possesses are no any toxic activity studied by in vitro models. The study also provides the use of this formulation is safe for the patients. Aconitum heterophyllum is a important ingredient of the formulation, some literature reveled that some Aconitum species are extremely poisonous in nature but I have taken the dried rhizomes of this plant it is nontoxic in nature and Ayurvedic formulation "Krshnadi Churna" It can be used as per indication of ayurvedic formulary of india futher it can be suggested that the in-vivo models can be taken for the studies of the formulation

DISCUSSION

In this in vitro toxicity assay, from table 6.1 & 6.2 and Fig. No. 3 and 4 it is observed the formulation of Krshnadi Churna. Shows non-totoxic activity against Hela cell line with IC50 value of 40.25 µg/ml for hydroethanolic fraction respectively by MTT assay. Vincristine sulphate is used as standard cytotoxic agent and the IC50 of test samples are compared with standard. The IC50 value of vicristine sulphate is 47.56 µg/ml. Parag R. Patel *et al*[1] done Cytotoxic activity for Rubia Cardifolia for different fraction against Hela cells and reported the significant to good cytotoxic activity, because Cytotoxic drug have toxicity Thus in relation to that result Krshnadi Churna formulation is having non- totoxic activity.

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