ANTI-CANCER ACTIVITY OF METHANOL EXTRACT OF *CINCHONA OFFICINALES* AGAINST SKMEL-3 CELL LINE (HUMAN SKIN CANCER)

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ABSTRACT

Studies were undertaken to evaluate the Anti-cancerous activity of Methanolic extract of *Cinchona officinales* against SKMEL-3 Cell line (Human Skin cancer). Leaves of cinchona sp. (Rubiaceae) are one of the familiar drugs for its therapeutic values in traditional as well as modern medicine. Even though a lot of research works has been carried out on cinchona alkaloids and its phenol constituents. Medicinal plants in the development of have a major role in the development of potent therapeutic values. The present study also evaluated the anti-cancerous activity of *Cinchona officinales*. Methanolic extracts were tested against SKMEL Cell line (human skin cancer cell lines) using 3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide or MTT) Assay. The cytotoxicity was confirmed by direct microscopic observation using Inverted phase contrast tissue culture microscopy, showed remarkable Cytotoxicity potential.

INTRODUCTION

Biodiversity is the basis of our existence on earth. Regardless of the benefits of biodiversity, today's threats to species and environment are among the greatest in recent history, virtually all caused by human mismanagement of biological resources. Conserving biodiversity is important to ensure intra- and inter-generational impartiality of Rainforests home to half ofthe world's fleas and animals. That is why the rainforest is called "The world's largest Pharmacy". Most modern medicines are derived from medicinal plants in the rainforest, which also have good biodiversity (**Bappenas et.al. 2003).** Many medicinal plants have become extinct from our ecosystem due to lack of know ledge about their medicinal properties' applications and benefits.

Cinchona Officinales is one of the most important medicinal plants widely distributed in South America and in 1859 this plant was introduced in India. This plant also spread in the east Andes and in the Amazon rain forest. The common name of this plant is Koina. In India cinchona plant is distributed throughout the Western Ghats (Nilgiri hills and Annamali hlls) and it is also grown in Darjeeling (west Bengal). Cinchona plant collectively known as koina

plant, Peruvian bark, Jesuits bark etc. This medicinal plant coming under the family Rubiaceae.. Hilly areas are suitable for the cultivation of this plant. Among all other countries Indonesia is largest producer of cinchona **(Kokate C.K., Purohit A.P et al).** There are 40 different plants are coming under the Cinchona species. The most important ones are Cinchona Officinales, Cinchona ledgeriana, Cinchona calisaya **(Widayat 2000).** Cinchona officinales plant is rich sources of alkaloids, more than 30 types of alkaloids present. Among these alkaloids Quinine is most effective and important alkaloid and is widely used as anti-malarial agent from ancient days. Many Literature study reports revealed that cinchona plant has many other medicinal properties like, Anti-bacterial, Anti-fungal, Anti-oxidant, Anti-cancerous, and Anti-inflammatory **(Megumi 2007).**

Numerous studies over the years have shown that cancer is one of the most dangerous diseases that spread not only to humans but also to animals and plants. Humans, plants and animals have suffered from cancerthroughout recorded history. Some plants have anticancer properties, using plants to treat cancer patients is considered a natural option. They may have properties that lower the risk of developing different types of cancer. When testing for various substances in plants known to treat cancer, as with any medical test, care ful safety precautions and considerations must be taken. Many medicinal plants have the ability to treat a wide range of common ailments **(Kupper T.S, Fuhl Briggs R.C,In 2004).**

Today skin cancer in humans increasing day by day several studies showed that UV radition and some other toxic substances cause the skin Cancer. The main reason for the skin cancer is the UV Radiation because of the ozone layer depletion **(Johnson T.M, Dolan O.M et al, 1998).** Various studies on the anti- cancer effect of cinchona showed that these plant alkaloids have the amazing anti proliferative activity against lot of cancers. Quinine alkaloid present in the cinchona officinales influences cell death and apoptosis in cancer cell lines and inhibits cancer cell proliferation in a dose and time dependent manner. . It has been established that quinine, which contains anti-cancer agents such as bleomycine and cisplatin, can increase or promote intracellular ROS generation (**Krishnaveni and Suresh, 2005).**

MATERIALS AND METHODS

The present study entitled anti-cancer activity of methanolic extract of *Cinchona Officinales.* AgainstSKMEL-3 Cell line (Human skin cancer). Different materials used and various methods adopted in the present study are described below.

Experimental plant: ***Cinchona officinales.***

Study Design

An experimental study was designed. This work was undertaken to examine the potential anticancer activity of Cinchona officinales against SKMEL-3 cancer cell line (Human skin cancer).

Experimental plant collection

Plant used in the study is Cinchona officinales and the plant leaves were collected from Medical plant Development Area (MPDA), Dodabetta, Doddabetta Essential oils & Herbs, Cinchona village, Ootty. 20grams of leaves collected and washed thoroughly with running tap water for 5 minutes. Washed leaves were dried above newspaper on the table for 1 week in room temperature. The newspaper was de- contaminated. After 1 week the leaves dried and shrinked. after that the dried leaves collected and kept it in hot air oven at 50celcious for 30 minutes and again the leaves collected from the hot air oven and the leaves were chopped and these were later grind into a powder with the aid of a grinder. The cinchona leaves were collected in a bottle and labelled. and kept the bottle in clean place and kept from water and other any contamination. The powdered plant material was then extracted.

Methanol Extract preparation

The entire plant's 25 gm of shade-dried coarse powder was packed tightly in a soxhlet device and subjected to continuous hot extraction with 150 ml of methanol until the extraction was finished. The solvent was totally removed from the extract by filtering it while it was still hot and distilling the resulting extract at reduced pressure in a vacuum, dried and maintained until experimentation in a desiccator. The obtained extract (a dark blackish brown colour)

was weighed, and the percentage yield of the air-dried powdered raw material (referred to as MTE) was computed.

CYTOTOXCICITY (ANTICANCER) SCREENING BY MTT ASSAY

Cell lines and maintenance: - SKMEL cell line (Human Skin cancer) was procured from National Centre for Cell Sciences (NCCS), Pune, India.

Cell culture media and maintenance: -

The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-Himedia), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and 1% antibiotic cocktail containing Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). The cell containing TC flasks ($25cm^2$) were incubated at 37^0C at 5% CO_2 environment with humidity in a cell culture incubator (Galaxy® 170 Eppendorf, Germany).

Cell preservation: - The cells were preserved at low passage number at liquid nitrogen vapor phase in modified cell culture media supplemented with 20% FBS and 10% DMSO or Glycerol.

Cell seeding in 96 well plates: -

80-90% confluent cells maintained in TC flasks were trypsinized. Trypsinization is the detaching process of adherent cells from a TC flask for sub culturing or for seeding 96 well plates for experiments. For this purpose, the monolayer of cells grown in TC flaks were exposed to Trypsin/EDTA solution (0.025% trypsin and 0.01% EDTA in Phosphate Buffered Saline). The trypsinized cells were diluted in the cell culture media at a concentration of 5×10^3 cells/well (in 100µl). The 96 well plates were seeded with cells and incubated for 3-4 days at cell culture incubator.

Sample Preparation and Treatment

The test sample was prepared in DMEM media (100mg/ml) and filter sterilized using 0.2 µm Millipore syringe filter. The Samples were further diluted in DMEM media and seeded to the wells containing cultured cells at final concentrations of 6.25 µg, 12.5 µg, 25 µg, 50 µg and 100 µg respectively. Untreated wells were kept as control. All the experiments were done in triplicate and average values were taken in order to minimize errors. After treatment with the test samples the plates were further incubated for 24 hrs

MTT ASSAY

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This colorimetric assay is based on the reduction of a yellow

tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan (Mosmann et al., 1983). The insoluble formazan crystals are dissolved using a solubilization solution (100% DMSO) and the resulting purple colored solution is quantified by measuring absorbance at 570 nm using an ELISA plate reader.

After sample treatment and incubation for 24 hrs, the media from the wells were aspirated and discarded. 100 µl of 0.5 mg/ml MTT solution in DMEM media was added to the wells. The plates were further incubated for 2-4 hrs for the development of formazan crystals. The supernatant was removed and 100 µLDMSO (100%) were added per well. The absorbance at 570 nm was measured with micro plate reader. Two wells per plate without cells served as blank. All experiments were done in triplicates.

RESULTS

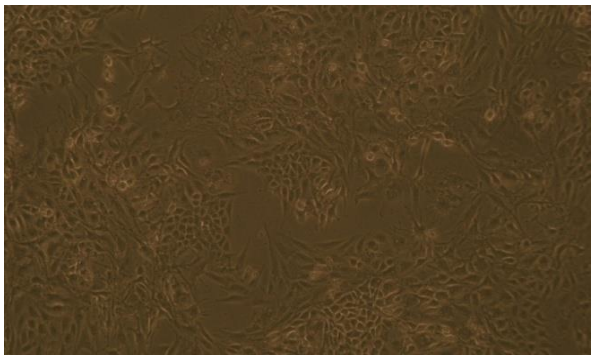
In vitro screening of cytotoxic compoundsInverted phase contrast Microscopy

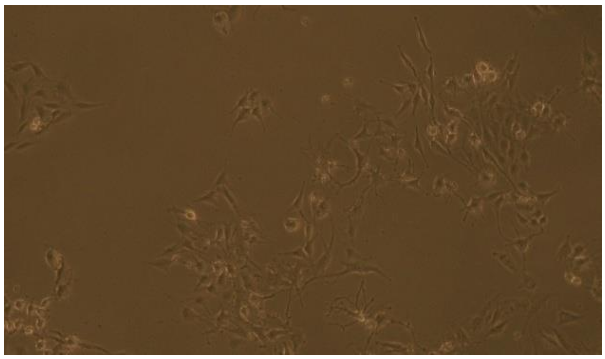
The viability of the test sample treated cells was evaluated by direct observation of cells by Invertedphase contrast microscope. The viability of the treated cells was further quantified by MTT assay method.

Direct microscopic observation

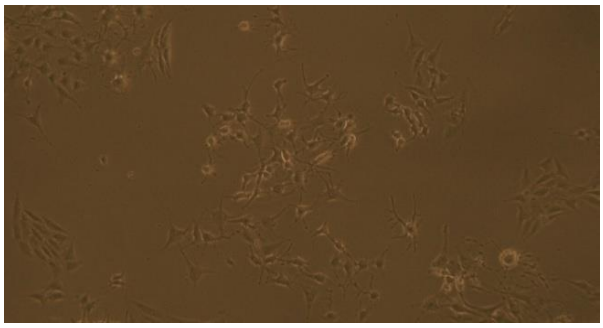
After sample addition, the treated as well as the control wells were observed at regular intervals up to 24hrs in an inverted phase contrast tissue culture microscope (Labomed TCM-400 with MICAPS™ HD camera) and the observations were photographed. Any detectable changes in the morphology of the cells,such as rounding, shrinking of cells, granulation and vacuolization in the cytoplasm were considered as indicators of cytotoxicity.

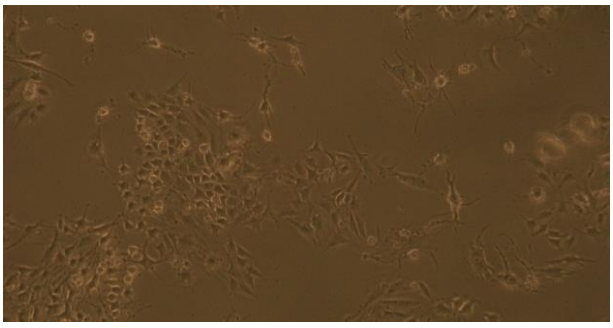
Morphological changes of cell lines:-round, shrinked cells indicate the presence of Cytotoicity.



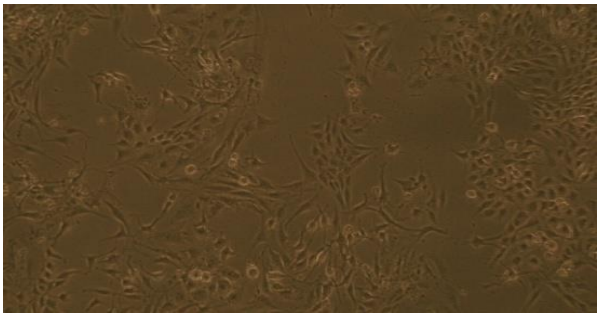


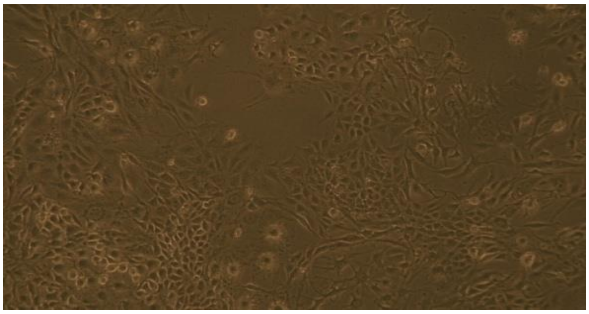
A. SKMEL cells without plant extract (Control cells) **B. SKMEL Cells treated with *C. officinales* (B-6.25 µg/ml)**





C.SKMEL Cells treated with *C. officinales* (12.5 µg/ml) **D**.SKMEL Cells treated with *C. officinales* **(25 µg/ml**





E. SKMEL Cells treated with *C. officinales* (50 µg/ml) **F. SKMEL Cells treated with *C. officinales* (100 µg/ml)**

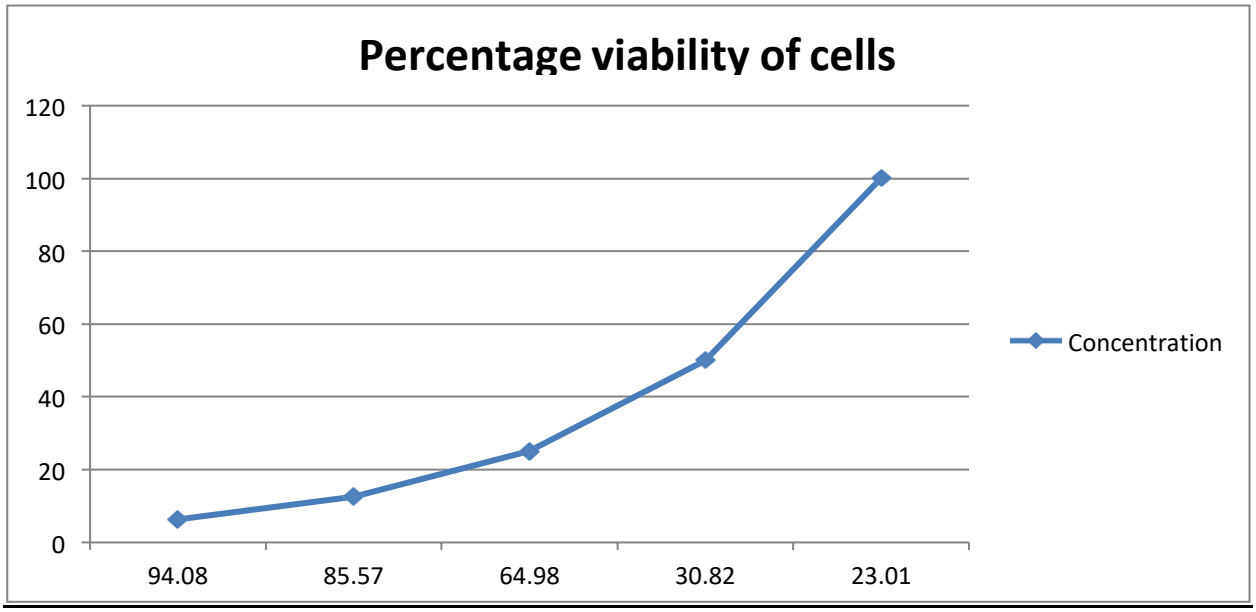
6.0 Anti-proliferative Effect of *Cinchona officinales* Extracts against SK-MEL-3 Cells

6.1 Table-2

	Sample		SKMEL	
	Response 1	Response 2	Response3	Average
Control	0.846	0.829	0.759	0.811
6.25	0.810	0.746	0.735	0.763
12.5	0.719	0.684	0.681	0.694
25	0.526	0.521	0.534	0.527
50	0.245	0.251	0.255	0.250
100	0.186	0.179	0.195	0.186
Concentration µg/ml	Percentage viability			

6.25	94.08			
12.5	85.57			
25	64.98			
50	30.82			
100	23.01			
Ic50	28.36			

Anti-proliferative effect of cinchona officinales against SKMEL-3 Cell line-MTT assay.



Percentage viability of cells

The cell viability was expressed using the following formula:

$$\text{Percentage cell viability \%} = \left(\frac{\text{Average absorbance of treated cells}}{\text{Average absorbance of control}} \times 100 \right)$$

IC 50- which means at what concentration at 50% of cells are dead, The IC 50 values were calculated using the equation for slope (y = mx + C) obtained by plotting the average absorbance of the different concentrations of the test sample (6.25 µg, 12.5 µg, 25 µg, 50 µg and 100 µg) in Microsoft Excel.

IC 50 **=28.36**

DISCUSSION

The result of Anti-cancer activity shows the presence of anti-proliferative effects present in the Methanolic extract. Detectable changes in the morphology of the cells, such as rounding, shrinking of cells, granulation and vacuolization in the cytoplasm were considered as indicators of cytotoxicity. In summary, our results indicate that naturally occurring plant components, including alkaloids and Phytochemicals may be used as starting structures for the potential development of novel anticancer agents.

CONCLUSION

Plants are known as the reservoir of secondary metabolites. From the beginning of the world medicinal values of plants was very famous in one or another way. Our today's world so many medicines have been developed from various herbal sources. Cinchona or the Peruvian bark is one of the major medicinal plant. Cinchona officinales commonly known for its anti-malarial activity and this plant also have some other medicinal properties like anti-cancer, anti-bacterial, anti- inflammatory etc. Cinchona plant can cure many diseases. One of the most significant medicinal property of cinchona is the presence of alkaloids. Over dose of cinchona may cause some diseases also. However cinchona considered as the Good medicine for many diseases.

The present study was undertaken to evaluate the Methanolic extract, of cinchona officinales to family Rubiaceae found moderate cytotoxic potential against SKMEL-3 skin cancer Cell line .Methanolic extract of cinchona officinales exhibited dose and time dependent killing capability against Human skin cancer cell line. Anti-cancer activity since it inhibited cancer cell development in skin .Our result suggest that Methanolic extract of cinchona officinales are promising anti-cancer reagents.

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