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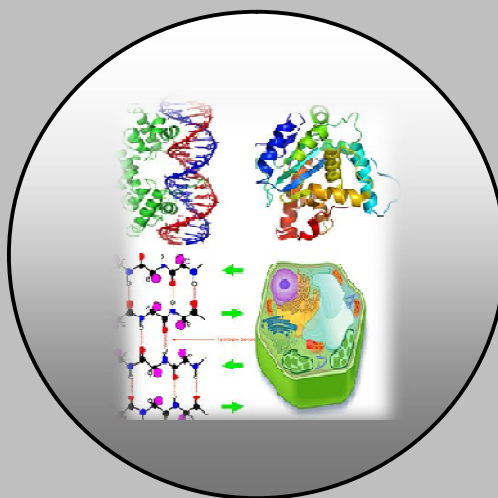
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RESEARCH PAPER

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Cytotoxicity Studies of *Chlorophytum borivillianum* against BHK-21 Cells

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ABSTRACT

Present study is to evaluate the anticancer property of methanolic, ethanolic and aqueous extracts of Chlorophytum borivillianum (Safedmusli) in BHK-21 cell lines. Aqueous extract of Chlorophytum borivillianum leaves was prepared as cold extraction method. Methanolic (MeOH) and ethanolic (EtOH) extracts were prepared in Soxhlet's apparatus and dried in vacuum evaporator. The BHK-21 cells were maintained in animal cell culture laboratory of the institute in GMEM media supplemented with 5% fetal bovine serum as per standard procedures. The cells were treated with each of the extracts in triplet wells of cell culture 24 well polystyrene plates and then allowed to grow for 24 hrs. The cell growth was inhibited within 24hrs; ethanolic and aqueous extracts showed better response. On microscopic examination clumping of cells, degeneration, rounding and shrinkage of cells were observed. The concentration of 10mg/ml of ethanolic extract showed higher anticancer activity.

Keywords: *Chlorophytum borivillianum*, BHK-21 Cells, and Anticancer Activity.

INTRODUCTION

It would not be an exaggeration to call *Chlorophytum borivillianum* (Safedmusli) a unique gift of nature to the mankind since the time immemorial. In the Ayurvedic literature, Safedmusli is celebrated as a Divya Aushad with unparalleled medicinal properties. It is a chief ingredient in the preparation of over a hundred Ayurvedic formulations. Safedmusli is also gaining increasing acceptance as a vitalizer and health-giving tonic, a curative for pre-natal and post-natal problems, a restorative for immunity-improvement and as a remedy for diabetes and arthritis (Mimaki *et al.*, 1996). The recent discovery of Safedmusli as a natural and safe aphrodisiac agent has also opened up new channels of application and new demand for it.

By virtue of being a herb, Safedmusli has been found to be an ideal aphrodisiac with no negative side-effects associated with chemical-based aphrodisiacs. As Safedmusli grows naturally in most parts of the Central Region of India, the normal climate of the Central region suits the crop most and it can grow successfully in the wide range of temperature and rainfall (Qiu, 2000). Sandy loam soil with proper drainage system facilitates its growth. Safedmusli is found growing in thick forests in its natural form such as Patwadangar near Nainital in Uttarakhand. It is partly a herb with sub-erect lanceolata leaves.

There are about 256 varieties of *Chlorophytum* and 17 among them are found in India. Among these, *Chlorophytum borivillianum* has good market both indigenously and globally. The Medicinal Plants Board has recognized Safedmusli as the sixth important herb to be protected and promoted. It is rich source of over 25 alkaloids, vitamins, minerals, proteins, carbohydrates, steroids, saponins and polysaccharides etc. A number of Health tonics (Sexual tonics) are prepared from Safedmusli (Rizviet *al.*, 2007). It is essential part of a traditional diet of mothers (after delivery) in the form of Laddoos. Efforts in countries like USA and England are also on to convert it into chips to use it as a nutritious breakfast. Its tubers roots are used in Ayurvedic medicines. Roots are used for the preparation of nutritive tonic used in general sexual weakness. There is a vast demand of Safedmusli all over the world. Tuberous roots of *C. borivillianum* also possess immunomodulatory properties and are used to cure impotency, sterility and enhance male potency. The main active principles of roots, saponins are stimulants and metabolic enhancers and have been shown to possess anti-tumor activity (Negiet *al.*, 1993).

The extract of dried root tubers of *C. borivillianum* acts apsycho-stimulant and has a beneficial effect on the brain and human body by increasing alertness, mental ability, intelligence and sexual characters. Due to its therapeutic activity and diversified uses, demand for *C. borivillianum* is increasing in Indian and the international market (Sebastian and Bhandari, 1984). The plant is also used for dysuria. Leaves are eaten by the tribal people of Western Ghats of India as an expectorant (Chetty and Rao, 1989). Root is used as aphrodisiac, diuretic, and astringent useful in dysentery, as an antidiabetic and as appetizing agent (Bhandaryet *al.*, 1995). Tubers are used as lactagogue (Sharma and Sharma, 1984). The plant is rich in many pharmaceutical active constituents, mainly glycoside which has been isolated from leaves (Tandonet *al.*, 1990) and roots (Brown *et al.*, 2006).

Although various researches have been done on various properties of *Chlorophytum borivillianum* this study is a step towards cancer prevention and it highlights on the mode of action of herbal plants in cancer therapy. A dose of chemotherapeutic drugs sufficient to kill tumor cells is often toxic to the normal tissue and lead to many side effects which in turn limit their treatment efficacy. Herbalism explains the unmistakable popularity of green magic bullets and is widely accepted in present perspectives. Plant-derived compounds comprise a diverse group with different mechanisms of actions, which seem to have the ability to induce apoptosis. Understanding the modes of action of plant derived anti-cancer compounds provide useful information for their possible application in cancer prevention (Moon *et al.*, 2001). It is thus important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them.

Medically screened plants which are used as traditional anticancer remedies increase the chance of finding new bioactive principles. Studies on the pharmacological mechanisms and searching for chemical structures from herbal extract for new anticancer drug caught great interest (Mudaiya *et al.*, 1987). Epidemiological studies have suggested that the medicinal plants can play a vital role in inhibiting the genotoxicity and carcinogenicity of anticancer drug to normal cells (Etukudo, 2003). Considering herbalism as an important strategy for cancer prevention, variety of animal experiments and cell cultures have been carried out. *Chlorophytum borivillianum* is a wild neglected herb which has various pharmacological properties viz. anti-inflammatory, antiallergic, antioxidant and immunomodulating activity. However, not much attention seems to be paid for trapping anticancer activity of this plant. The phytochemicals like flavonoid, isoflavonoid, polyphenol etc., have garnered great interest for their potential role in the maintenance of human health in particular, significant cancer risk reduction (Robert *et al.*, 2000). Considering the importance of diverse medicinal properties of *Chlorophytum borivillianum*, the present study has been planned to carry out *in vitro* studies on its anticancer properties on BHK-21 cells.

MATERIAL AND METHODS

Collection of Material and its Processing

Plant has been selected from high altitude area (1600m from sea level) from the poly house nursery of Institute of Biotechnology, Patwadangar, Nainital and leaves of *Chlorophytum borivillianum* were collected (Fig.1) and were washed with tap water thrice. Washing was again repeated five times by using distilled water. Then the leaf samples were air dried and thereafter kept in incubator at 37°C for 24 hrs. The dried plant material was then crushed in mechanical grinder in order to make fine powder which was stored at room temperature for further use.

Extract Preparation

Aqueous Extract

The aqueous extract was prepared according to the standard method with slight modifications. Now, 5 g of leaf powder was mixed in 120 ml of water and was kept in incubator shaker at 36°C and 100 rpm. The extract so obtained was evaporated to drying through heating in a china dish. Dry extract was then scrapped off, weighed and reconstituted in normal saline (Marks *et al.*, 2000).

Solvent Extraction

Ethanol and methanol extracts were prepared in Soxhlet's apparatus. Soxhlet's extraction was carried out at room temperature. Dried Leaves of *Chlorophytum borivillianum* weighed accurately 5gm and taken in thimble and subjected to extraction in a Soxhlet's apparatus at room temperature using ethanol (150ml) and methanol (165ml) (Govindachari *et al.*, 1999). The extract obtained was first filtered using Whatman No. 1 filter paper and solvent was then removed under reduced pressure in a vacuumed rotary evaporator and dried. The dried extract was stored in air tight containers for further studies.

For the present study, BHK-21 cells were cultured in 24 well sterile polystyrene plates using GMEM media supplemented with 5% fetal bovine serum as per standard procedure. The cells were seeded into 24 well sterile polystyrene plates and were incubated for 24 hours at 37°C. Thereafter, the medium was removed and 0.5ml of each dilution (10mg, 1mg, 10µgm) of each extracts added to the assigned wells, Control well were also kept (medium without test sample) and triplicate sets of each dilution were maintained. Finally the cells were incubated for 24 hours at 37°C and thereafter, examined under inverted microscope for their morphological studies.

MTT Assay

The MTT Assay is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) into a dark formazan product that is insoluble in water.

The amount of formazan produced is directly proportional to the cell number in a range of cell lines. It was performed as, prepared an MTT stock solution of 5 mg ml⁻¹ in phosphate-buffered saline (PBS), pH 7.5, and filter through a 0.22-µ filter to sterilize and the small amount of insoluble residue was removed. Add 10 µl of MTT (5mg ml⁻¹), after 24 h of incubation and the cells were further incubated in incubator at 37°C for 3 h. Then 100 µl 0.04 M HCl in propan-2-ol to each well were added and mixed thoroughly to dissolve insoluble blue formazan crystals. The Plates were read on a micro-ELISA reader using a test wavelength of 570 nm (Mosmann, 1983).

Neutral Red Assay

Neutral red (3-amino-7-dimethyl-2-methylphenazine hydrochloride) is a water soluble, weakly basic, supravital dye that accumulates in lysosomes of viable cells. The neutral red (NR) assay is an *in-vitro* cell viability test that was developed and extensively studied for *in vitro* cytotoxicity determination. After incubation of cells with extracts, 0.33% of NR (NR in PBS) was added in each well and incubated for 1 h at 37°C. Dye-containing medium was removed and the well was washed twice with 150 µl/well warmed PBS. The cells were then lysed with 125 µl of 50% of v/v mixture of ethanol and 0.1M monobasic sodium phosphate to solubilize the neutral red. The plate was then incubated for 15 min and take O.D at 550 nm (Flick and Gifford, 1984).

Cytotoxicity % = $A-B/A \times 100$

A = O.D of untreated well; B = O.D of wells treated with plant extract. The data so obtained were calculated and were presented in table-1 & 2 to find out %inhibition

RESULTS AND DISCUSSION

The cells were observed after 24hours to record the changes in morphology (Fig.2). The induction of apoptosis resulted in cell shrinkage, eventually leading to the formation of apoptotic bodies. As are result of cytopathic effect the cells also got detached from the substratum shown by flagging of the cells and also they forms clump after detachment from the substratum.

The morphological changes clearly revealed that the aqueous and methanolic extract of *Chlorophytum borivillianum* (Safedmusli) leaves were better than the ethanolic extract of leaves. The shapes of cells are changed (Fig.3). Elongated cell turn to round cell and clumping was observed. The cytotoxicity study was also clearly indicated, that the aqueous and methanolic extract of *Chlorophytum borivillianum* (Safedmusli) kill more cells then that of ethanolic extract.

Cytotoxicity measured by MTT Assay of ethanolic and aqueous extract of *Chlorophytum borivillianum* (Safedmusli) proves better than that of its methanolic extract (Table 1). The cytotoxicity at 10 mg/ml was 76% in ethanolic extract of *Chlorophytum borivillianum* (Safedmusli), it was 73% in aqueous extract of *Chlorophytum borivillianum* (Safedmusli) at same concentration but the methanolic extract at this concentration was 69%. At 1 mg/ml the cytotoxicity of aqueous extract and ethanolic extract were 70% and 75%, respectively and at the same concentration the methanolic extract give 67% cytotoxicity. At the lowest concentration of 10µgm/ml the aqueous and ethanolic extract showed 71% and 72% cytotoxicity respectively, and the methanolic extract show 66% cytotoxicity.



Fig 1. *Chlorophytum borivillianum* (Safedmusli)

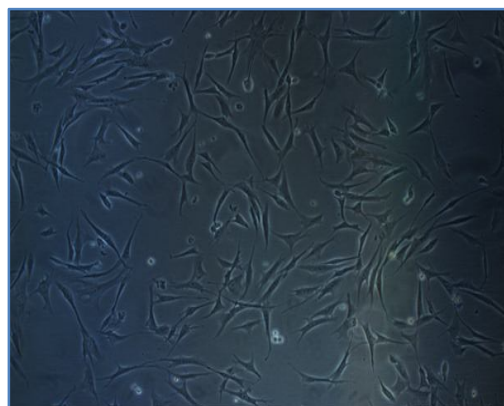


Fig 2. Normal BHK-21 cells.

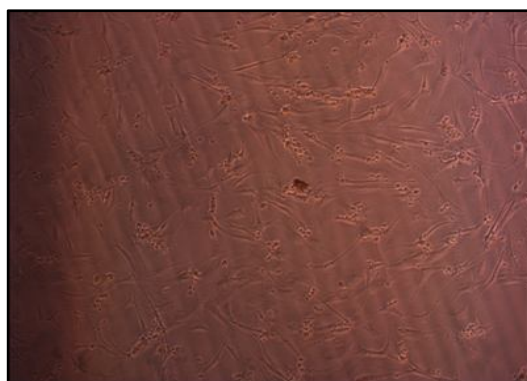


Fig 3. BHK-21 Cells after Treatment with Extracts.

Table 1. Percent Reduction in Cell Growth due to Extracts of *Chlorophytum borivillianum* leaves as Measured by MTT assay.

Sl.No.	Extracts	10mg/ml	1mg/ml	10µgm/ml
1.	Aqueous extract	73.21±2.61	70.13±2.51	71.82±2.14
2.	Methanolic extract	69.59±2.13	67.86±2.78	66.89±2.29
3.	Ethanolic extract	76.41±2.42	75.67±2.51	72.58±2.34

Table 2. Percent Reduction in Cell Growth due to Extracts of *Chlorophytum borivillianum* leaves as Measured by NR assay.

Sl.No.	Extracts	10mg/ml	1mg/ml	10µgm/ml
1.	Aqueous extract	75.41±3.26	74.07±3.12	72.11±3.19
2.	Methanolic extract	68.91±2.84	66.42±2.71	64.31±2.59
3.	Ethanolic extract	78.20±3.03	76.31±3.17	74.22±3.23

Cytotoxicity measured by Neutral Red Assay of ethanolic and aqueous extract of *Chlorophytum borivillianum* (Safedmusli) showed better response than that of its methanolic extract. (Table 2) The cytotoxicity at 10 mg/ml was 78% in ethanolic extract of *Chlorophytum borivillianum* (Safedmusli), it was 75% in aqueous extract of *Chlorophytum borivillianum* (Safedmusli) at same concentration but the cytotoxicity of methanolic extract of *Chlorophytum borivillianum* at this concentration was 68% which was less than that of its aqueous and ethanolic extracts. At 1 mg/ml the cytotoxicity of aqueous extract and ethanolic extract were 74% and 76%, respectively and at the same concentration the methanolic extract give 66% cytotoxicity. At the lowest concentration of 10 µgm/ml the aqueous and ethanolic extract showed 72% and 74% cytotoxicity, respectively and the methanolic extract showed 64% cytotoxicity.

The scientific evidences collected from the study supports the traditional claims behind usage of the herb *C. borivillianum*, which is being cultivated and marketed extensively in India and abroad for medicinal purposes. The study affirms that *C. borivillianum* leaves extract was having effective cytotoxic property. The inference that can be drawn from the present study was that the aqueous and ethanolic extract were superior over methanolic fraction of the plant as far as anticancerous effectiveness of extracts activity is concerned. From the earlier studies, it was confirmed that the extract does not only potential paraspecific immune response, but was also effective in improving humoral as well as cell-mediated immunity (Kothari, 2004). Use of herbs for improving the overall resistance of body against common infections and pathogens has been a guiding principle of Ayurveda (Patwardhan *et al.*, 2005). *Chlorophytum spp.* have been used and reported in many such formulations.

On the basis of observed encouraging cytotoxic effects by the *in-vitro* bioassays, it can be revealed that ethanolic and aqueous extracts of *Chlorophytum borivillianum* (Safedmusli) leaves has promising anticancer bio efficacy and must have some chemical moiety in the leaves of this plant which might be responsible for observed beneficial effects.

It is suggested that the detailed *in-vivo* studies should be carried out in animal experimental model of cancer to further prove the anticancer activity of *Chlorophytum borivillianum* (Safedmusli).

CONCLUSION

Ayurvedic medicine is a system of traditional medicine native to Indian subcontinent and is considered original system of medicine. Ayurveda that subsided for sometime has now made a comeback. Ayurvedic herbs and the medicinal plants are a key component of Ayurveda, the traditional medicine of India. In general, practitioners use Ayurvedic herbs to cleanse the body, boost defense against disease, and keep the mind, body, and spirit in balance. Along with Ayurvedic herbs, practitioners frequently use certain therapeutic oils and spices to treat illness and promote well-being. Several medicinal plants are under investigation to discover drugs for various diseases especially the dreadful ones that still await proper medication. In this study a plant was studied *Chlorophytum borivillianum* (Safedmusli) which was earlier known for many medicinal properties. The aqueous, ethanolic and methanolic extracts were tested on BHK-21 cells; which showed cytotoxic effects on the cancer cells. The leaves of *Chlorophytum borivillianum* (Safedmusli) can be further tested in *in vivo* model which may be a good candidate in anticancer therapy in future.

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