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Research Article

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In vitro Anticancer Activity of Ethanolic Extract of *In vitro* and *In vivo* Tuber of *Ceropegia Pusilla* Wight and Arn.

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ABSTRACT

The present work deals with the antiproliferative activity of in vitro and in vivo tuber against the HeLa cancer cell line and to analyses the phytochemicals present in the ethanolic extract of Ceropegia pusilla in vivo and in vitro tubers. Quantitative phytochemical analysis of in vivo and in vitro tubers confirm the presence of alkaloid in ethanolic extract. Among the different concentration of extracts used for antiproliferative activity at 50 µg/ml concentration showed the maximum cytotoxic effect by the increased number of dead cells ie 85 %, 82% respectively.

Key words*: Phytochemicals, Cytotoxic, Ceropegia pusilla and Antiproliferative.*

INTRODUCTION

Historically, plants have provided a source of inspiration for prominent drug compounds, as plant derived medicines have made large contributions to human health and wellbeing. Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world. Moreover, over 50% of all modern clinical drugs are of natural product in origin[1].

Phytochemicals are responsible for medicinal activity of plants. Phytochemical constituents are the basic source for the establishment of several pharmaceutical industries. The constituents are playing a significant role in the identification of crude drugs[2]. Angiogenesis, the formation of new blood vessels is a biological process that plays a fundamental role in embryonic development[3]. It plays a critical role in various physiological and pathological process such as embryonic development, wound healing, chronic inflammation, tumor growth, and metastasis[4,5]. Angiogenesis blockade has been shown to be an effective strategy in inhibiting tumor growth and metastasis[6]. Consequently, cytotoxic agents pose as candidates as antiangiogenic agents on top of their potent activity in causing death of cancerous cells. Studies have been conducted to assess the role of oxidative stress and hence the use of antioxidants in the prevention of many diseases such as cancer, inflammation, and atherosclerosis[7,8].

The family Asclepiadaceae, also known as milkweed family due to the presence of milky latex in its members has lot of medicinal uses[9]. The genus, *Ceropegia* is under threat owing to either destructive collection or habitat degradation. Fifty species are present in India[10], out of which 28 species are endemic to Peninsular India[11,12]. The tuber of this plant is edible and contains "cerpegin'' alkaloid[13]. The tuber of *Ceropegia pusilla* is used in Ayurvedic preparations that are active against many diseases like diarrhea, dysentery and syphilis. Tubers contain nutritive starch and it has tonic and blood purifier[14,15]. This work has been undertaken to investigate the anticancerous activity and phytochemicals of *C.pusilla* ethanolic extracts of *in vivo* and *in vitro* tubers.

MATERIALS AND METHODS

In vivo tuber

Plant of *Ceropegia pusilla* were collected from Ooty, Nilgiri District., Tamilnadu (India), and authenticated by Botanical Survey of India (BSI/ SRC/ 5/23/2012-13/ tech 1268) Coimbatore. The plants were grown in earthen pots in shade house at Government Arts College, Coimbatore.

In vitro tuber

The *Ceropegia pusilla* microtubers formed in MS medium containing BAP (1.5mg/l) and NAA (0.5mg/l) was collected from our tissue culture lab[16].

Preparation of Extract

The *in vivo* and *in vitro* fresh tuber were carefully washed with tap water, rinsed with distilled water, and air dried for one hour. Then it was cut into small pieces, dried in room temperature for two weeks and powdered in a local mill. Two grams of each pulverized plant material was extracted by maceration with ethanol for one week. The extracts were concentrated in vacuum (Rota vapor) and the residues from the ethanol extract were weighed and stored in sealed vials in a freezer until tested.

TLC -analysis of identification of alkaloid

Thin layer chromatography is particularly valuable for quantitative determination of small amount of phytochemical compounds present in plant samples. The method of Wagner *et al.,*[17] was adopted for the analysis of alkaloids using TLC.

Preparation of TLC plates

The glass plates to be coated were cleared thoroughly, free of any grease and wiped with alcohol and dried in special racks. They were arranged on the applicator pad with a few drops of water used as a means for dicing these plates immobile on the pad.

Coating materials

The weighed absorbent silica gel-G (50g) was mixed with 100ml of distilled water either by grinding in a mortar or by vigorous shaking for 10 minutes in a stoppered conical flask. Later it was poured quickly to the spreader to have a uniform layer.

Coating the plates

The aperture-opening in the applicant was set for desired thickness of the coating (usually 250µ). The slurry silica gel-G was poured into the applicator placed on the last plate on the left of the pad. The lever was turned into 180°C. When the slurry could be seen coming out, the applicator was drawn smoothly on the plates.

Drying and Storing

The coated plates were left in the same position overnight. When necessary, these plates were activated after drying in a hot air oven at 105ºC for 30-60 minutes. The plates were placed in special racks provided, to prevent the layers from chipping off. Since these active plates are easily deactivated by moisture, the plates were stored in a desiccator till use.

Application of the samples

Extract was dissolved in suitable solvent. TLC plate was placed horizontally on a white paper marked with a base line. Sample was applied on the line in the form of spot, drop by drop with the help of capillary with regular interval. Authentic samples were also applied simultaneously in the same manner. The plate after application of sample was kept inside the chromatographic tank contained mobile solvents (Chloroform: methanol: Ammonia (aqueous) (75: 45:1.5) in such a way that the margin of the plate nearer to the baseline just touched the solvents. It was then covered with a glass plate and left for 2-4 hours at the room temperature. The separation of fractions was carried out with single dimensional TLC.

Detection of chromatogram

Alkaloids: The developed plates were air-dried and sprayed Dragendroff' reagent. After spraying, the plates were heated at 100°C for 5 min until characteristic colors developed. The presence of the compounds was confirmed by the presence of bands after spraying with respective reagents. All tests were repeated thrice.

***In vitro* cytotoxic assay**

This assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based 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-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water.

Plating out of cells for 96-well plate cytotoxic assay

Trypsinize a sub-confluent monolayer culture, and collect the cells in growth medium containing serum was centrifuge the suspension to pellet the cells. Re-suspend the cells in growth medium, and counted them the seed 5000 cells per well of 96 well plate. Added 200 µl of growth medium to the eight wells in columns 1to 12. Column 1 will be used to blank (without cells) the plate reader.

The incubate the plates in a humidified atmosphere at 37^{o}C for 24 hr, such that the cells are in the exponential phase of growth at the time the drug / toxicant is added and remove the growth medium using pipette. Take care that the tip of the pipette does not touch the cell sheet and wash the cell monolayer once with PBS. Then add required volume of growth medium to the cell sheet and add medium containing defined concentrations of the drug followed the same for solvent control add 200 µl of the medium free from the drug to the control wells were incubate the plates at 37^{o}C in 5% CO_2 environment for required time points.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was done using the method of Yuan *et al.* (2004). The cells in suspension containing approximately 1×10^6 were added to each well of a 96-well culture plate and were incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HeLa cells were treated with various concentrations 10µl, 20µl, 30µl, 40µl and 50 µl of ethanolic *in vivo* and *in vitro C.pusilla* tuber extracts. Control cultures were treated with DMSO. The cultures were again incubated as above. After 36 h, 20 µl of MTT solutions were added to each well and the cultures were further incubated for 4 h and then 200 µl of DMSO was added. The formed crystals were dissolved gently by pipetting two to three times slowly. The absorbance at 655 nm was measured using plate reader. The percentage of cell viability was calculated[18]. The samples without extract (viable cells with MTT) was used as control. Cyclophosphamide is used as positive control. Cultures were viewed using an inverted phase contrast microscope.

Analysis of data

The absolute value of the absorbance should be plotted so that control values may be compared, but the data can then be converted to a percentage-inhibition curve, to normalize a series of curves. The percentage inhibition is calculated, from the data, using the formula

= Control OD - Test OD / Control OD X 100

RESULTS AND DISCUSSION

Preliminary qualitative analysis of bioactive compound revealed the presence of alkaloid in ethanolic extract and shown spot on TLC after applying Dragendroff reagent (Plate 1).

The results of *in vivo* and *in vitro* tuber ethanolic extract of *C.pusilla* against HeLa cell line exert antiproliferative action (Fig 1). The biochemical feature character of loss of viability was exhibited in dying cells. The morphological changes were observed under microscopy. In 30 -50 µg/ml concentrations, after 5-6 h of treatment some of the cells were beginning to separate from the plate and

become rounded. Different levels of cytotoxicity like cell rounding, aggregation, shrinkage and cell death was observed depending on the concentration of the extracts (Plate 1). In both tested samples cytotoxic effect was observed with the evidenced by increased number of death cells, 85.0%, 82.0% respectively at the concentration of 50 µg/ml (Plate 1 E &C).

The medicinal and pharmacological actions of medicinal herbs are often depended on the presence of bioactive compounds, the secondary metabolites[19]. The use and search for, drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, biochemists, botanists and natural-products chemists all over the world are currently investigating medicinal herbs for phytochemicals and lead compounds that could be developed for treatment of various diseases[20].

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Medicinal plants represent a vast potential resource for anticancer compounds. The anticancer activity of medicinal plant derived compounds may result from a number of mechanisms, including effects on cytoskeletal proteins that play a key role in cell division, inhibition of DNA topoisomerase enzymes, antiprotease or antioxidant activity, stimulation of the immune system etc. The value of medicinal plants lies in the potential access to extremely complex molecular structures that would be difficult to synthesize in the laboratory[21]. The limitations of the available cancer management modalities create an urgent need to screen and generate novel molecules. Despite, well-documented illustrations of phytochemicals being used for prevention and treatment of cancer, their importance in modern medicine remains underestimated. Plants are the storehouse of "pre-synthesized" molecules that act as lead structure, which can be optimized for new drug development. In practice, a large number of cancer chemotherapeutic agents that are currently available in the market can be traced back to their plant resource[22].

In vivo and *in vitro* ethanolic tuber extracts of *C.pusilla* were tested for their antiproliferative activity. *In vitro* antiproliferative property of tubers of *C.pusilla* confirmed by the present results. When compared to *in vivo* tuber extract the *in vitro* tuber extract also effective in inducing cytotoxicity. Morphological analysis of the cells exhibited that the extract treatment had initiated apoptotic mechanism to trigger cell death. At 50 µg/ml concentration over 85%, 82% of the cell population had been rendered apoptotic by both *in vivo* and *in vitro* respectively through slow cytotoxic observation. The anticancer activity has been evidenced against a panel of cell lines including human epidermal carcinoma of the nosopharynx, COLO-320 and HeLa[23]. For the first time this study demonstrates that cell cycle disruption is taking place on the basis of antiproliferative action of the tuber extract of *C.pusilla*. Blocking the cell cycle at S phase and prevented the cells from entering the proliferative phase is the inhibitory effect of concentrations of extracts. A large number of phytochemical have been shown to inhibit cell cycle progression of various cancer cells[24].

CONCLUSION

Qualitative phytochemical analysis of *in vivo* and *in vitro* tuber confirm the presence of alkaloid in ethanolic extract and shown spot on TLC after applying Dragendroff reagent. *In vivo* and *in vitro* tuber extracts of *C. pusilla* confirms the *in vitro* antiproliferative property against HeLa cancer cell line. A comparison of antiproliferative activity of the extracts reveals that both *in vivo* and *in vitro* tuber extracts were effective in inducing cytotoxicity. Different levels of cytotoxicity like cell rounding, shrinkage, aggregation and cell death, depending on the concentration of the extract was observed in all the concentration. At the concentration of 50 µg/mL, both the tested samples produced cytotoxic effect as evidenced by the increased number of dead cells (85%, 82% respectively).

Our study, however, is the first report on the phytochemical analysis and antiproliferative property against HeLa cancer cell line of *in vitro* regenerated tuber organs as compared with *in vivo* tuber organs. In addition, our results confirm that *in vitro* tuber was effective in the same way as *in vivo* tuber. From this study we confirmed that the *in vitro* tubers can be used for medicinal purpose without disturbing the natural habitat.

Fig.1- MTT Assay or cytotoxic activity of the ethanolic extract of *Ceropegia pusilla in vivo* and *in vitro* tubers

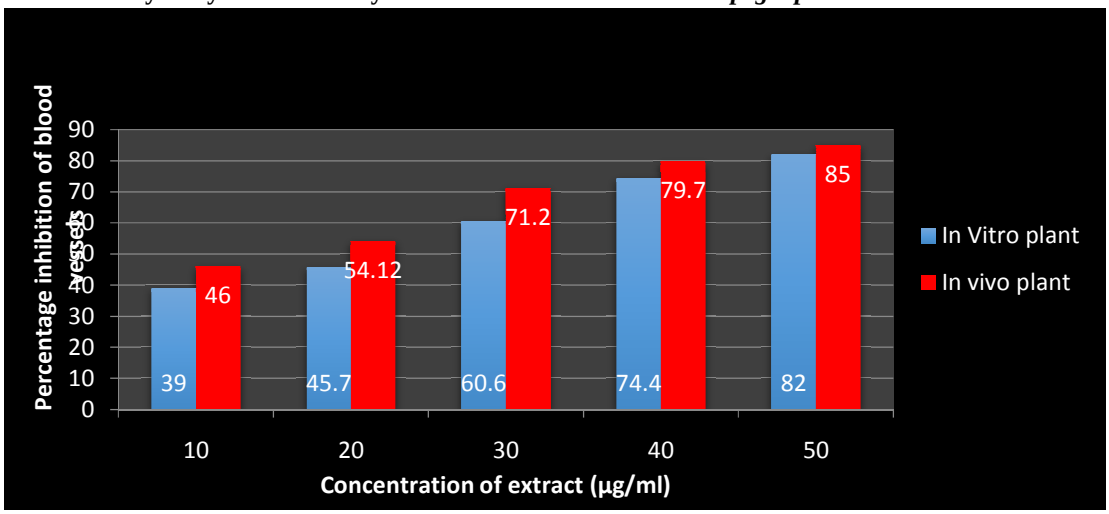
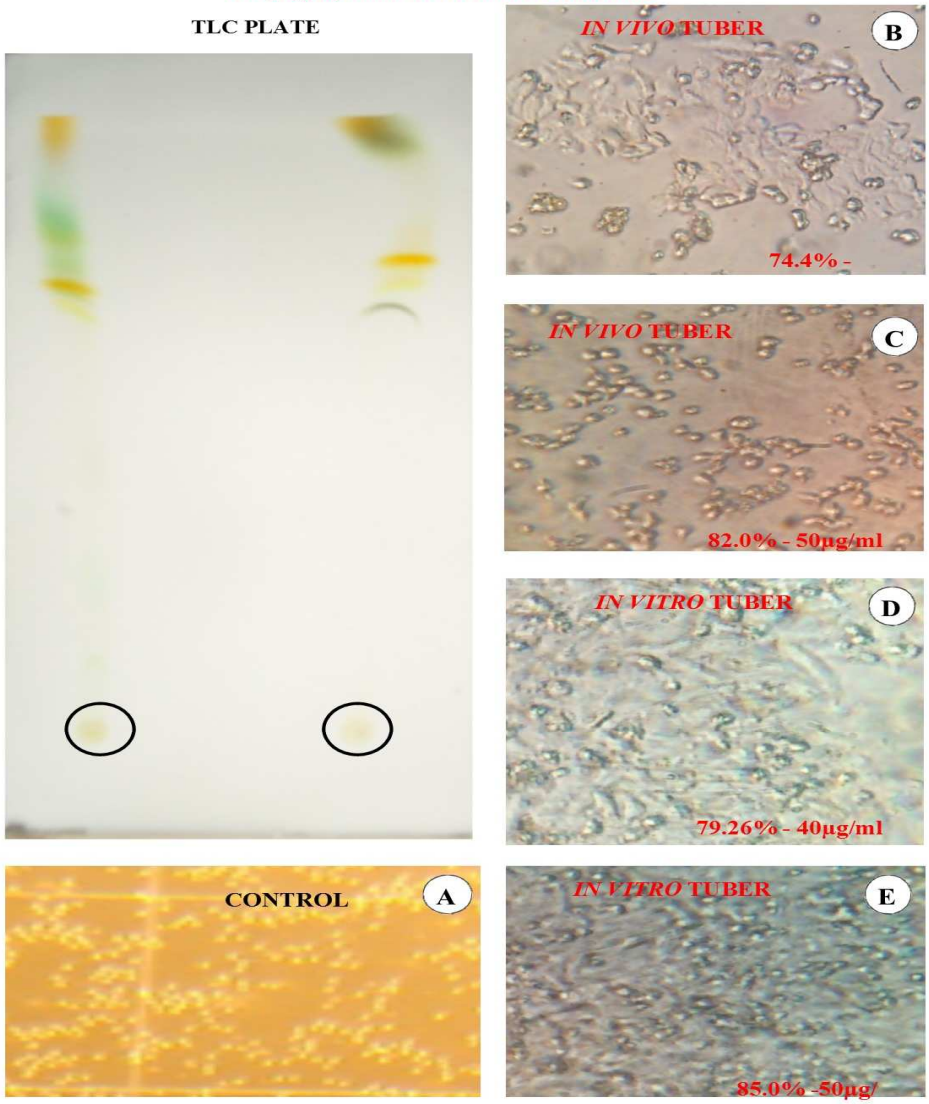


PLATE -1. MTT Assay (Cytotoxic activity) of the ethanolic extracts of *Ceropegia pusilla in vivo* and *in vitro* tuber



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