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In vitro anti-cancer activity of Piper betel leaf extract on HA -29 and its anti-oxidant activity

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ABSTRACT

Wayanad is a land with a luxury of flora and fauna. These repositories could be utilized by the reaserchers those who are interested. Here we used the Piper betel leafs collected from Wayanad district, Kerala against Colon cancer which is one of the best understood neoplasms from genetic perspective. Yet it remain the second most common cause of cancer related death, indicating the some of its cancer cells are not eradicated by current therapies. It is reported that some herbs protect the body from cancer by enhancing detoxic functions of the body. The detoxification, anti-oxidation, anti-mutation, anti-diabetic, cardiovascular, anti-inflammatory / immunomodulatory, anti-ulcer, hepato-protective and anti-infective properties of betel leaves also explored. In this study we examined inhibition of HA- 29 cell proliferation by piper betel leaves. The LD 50 value 98.4442µl/ml possess good anticancer activity against colon cancer cell lines (HT- 29). The anti-oxidant activity of piper betel leaf extract also analyzed.

Keywords: *cancer, medicinal plants, cell lines.*

I. INTRODUCTION

Nature is a repository of remedies for many of the health care issues. The importance has been thoroughly studied and reported in diverse aspects for natural compounds derived from diverse origin especially plants. The abundance and miscellany of plants and the relatively negligible side effects has been the marked features of plants with medicinal properties. There are many plants and plant origin compounds widely employed for treatment of many life threatening diseases like cancer but without proper documentation and scientific supporting evidences. The traditional knowledge regarding the medicinal property of a unique variety of plant has been kept as a secret among a limited number of individuals and possibility of losing the information without proper sharing may result in the loss of data forever. Moreover deforestation and the profit based human interventions would cause the devastation of some invaluable plant varieties from the universe. There is an urgent need for proper documentation and preservation of medicinally important plants across the globe for a better survival of human being in the light of rapid rate of deforestation and loss of biodiversity.

Betel leaves are in common use for centuries as a chewing element. There are different varieties and *Piper betel* is a well known vine belongs to the Piperacea family of plants. These *Piper betel* leaves have immense medicinal properties which is inherent to the biochemical composition and functional capacities. The composition and concentration of the piper leaves vary according to season, plant variety and climate. They are known to use as an agent for treatment of oral cancer owing their ability to maintain the concentration of ascorbic acid in saliva. We know that ascorbic acid is an evident antioxidant, which may help to reduce and control the free radical accumulation in the body. This unique antioxidant feature of piper betel leaf is being exploited for using them for cancer treatment especially oral cancer. In addition to this, bactericidal and aphrodisiac properties are also being reported for the piper leaf.

Chewing them may reduce the load of bacteria in mouth causing the bad breath and hence much popular among the common people. The gastro protective property of the betel leaf extract has also been reported (Dinesh *et al.,* 2016) and exploration of the still inherent but unidentified properties of the leaf extract would be of great concern from a public health viewpoint. Here in this study we investigate the ant oxidant and anti cancer properties of betel leaf extracts in different cell lines by in vitro.

2. Materials and methods

2.1. Collection of Medicinal Plants and extract preparation

Medicinal plants *Piper betel* leafs commonly known as betel leafs collected from in and around Wayanad Region, Kerala, South India. The plant was identified genuinely from Vanamoolika Herabal research Foundation, Wayanad and the extracts were prepared by the method of Uhegbu *et al*. (2005) using distilled water as the solvent.

2.2. Phytochemical Studies of Plant Extracts

The crude plant extract (Hot and Cold) was subjected to phytochemical analysis for noticing the chemical composites in it.

2.3. Anti-oxidant properties of Betel leaf extract - Phosphomolybdenum assay (Total antioxidant capacity)

Total antioxidant activity of the extract was evaluated by phosphomolybdate method using ascorbic acid as standard. The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/MO (V) complex at acidic Ph. An aliquant of 0.1 ml extract was combined with 3ml of reagent solution (0.6 M Sulphuric acid, 28Mm sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 degree C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695nm against the blank. Methanol (0.1 ml) in the place of extract is used as the blank. Ascorbic acid equivalent were calculated using standard graph of AA. The experiment was conducted in triplicates and values were expressed as the equivalent of ascorbic acid per mg of extract.

2.4. In vitro Anti proliferative effect and cytotoxic determination by MTT assay

The anti- cancer activity of *Piper betel* leaf extracts on HT- 29 was studied in Biogenix research centre, Trivandrum, Kerala in order to determine the cell viability after plant extract introduction that measures membrane integrity and effect of the plant extract on cell growth.

The cell line was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Dulbeccos modified Eagles Medium (EMEM) containing 10% fetal bovine serum (FBS). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were maintained at 37^0 C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week (Mosmann, 1983; Monks *et al*., 1991).

2.4.1. Cell Treatment Procedure

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. 1 mg of *Piper betel* Lyophilized leaf powder was added to 1ml of DMEM and dissolved completely by cyclomixer. After that the extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

After 24 hours the growth medium was removed, freshly prepared plant extracts in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 100µl of 5% MEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

2.4.2. Cytotoxicity Assay by Direct Microscopic observation:

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and

vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

2.4.3. Cytotoxicity Assay by MTT Assay Method:
Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilise the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 570 nm (Laura B. Talarico et al., 2004). The percentage of growth inhibition was calculated using the formula:

% Cell viability = Abs (sample)/Abs (control) x100.

3. Result

The medicinal plants were collected from in and around wayanad region and hot and cold extracts were prepared using distilled water as solvent. Preliminary screening and identification of bioactive chemical element in the *Piper betel* were carried out in both extracts. (Table - 1)

The quantitative phosphor molybdenum method was utilized to evaluate the total anti-oxidant capacity of the Piper betel leaf extract. The reducing power of the compound is associated with electron donating capacity and serves as an indicator of anti-oxidant activity with different in the digree of M0 reduction between extract used .Experiment indicated Piper betel leaf extract showing high degree of anti-oxidant capacity than the ethanol extract.

The anticancer activity of *Piper betel* leaf extracts on colon cell lines were studied in Biogenix Research Centre Trivandrum, Kerala. Addition of higher concentration of leaf extract, the viability of cells reminded almost as the primary concentration. The LD- 50 value was calculated as 98.4442µl/ml (colon cell lines) of extracts needed for the 50% of cell death. Results indicated that Piper betel extract had significant activity against on cancer cell lines. (Table – 2).

Table: 1 - Phytochemical result

Tests	Extracts (Piper betel leafs)	
	Hot extract	Cold extract
Saponin	–	–
Tannin	–	–
Anthraqunone	–	–
Flavanoid	+	+
Salkowsky	–	+
Phenol	–	–
Amino acids	+	+
Sugars	–	–
Reducing sugars	–	–
Proteins	–	+

Abbreviation: - + (Positive**) - (**Negative)

Table- 2: In vitro anticancer activity of *Piper betel* leaf extracts against colon cancer cell lines (HT29) - Percentage of cell viability (MTT Assay). The % Cell viability was determined by using the following formula. % Cell viability = Abs (sample)/Abs (control) x100.

Sample volume (µl)	Average OD at 540nm	Percentage Viability
Control	1.4526	
SAMPLE - B		
6.25	1.3864	95.44265
12.5	1.3271333	91.36261
25	1.2343	84.97177
50	0.9623	66.24673
100	0.7574	52.14099

LD 50 value – 98.4442µl/ml (ED50plus software v1.0)

4. Discussion

Colon cancer is one of the best understood neoplasms from genetic perspective. Yet it remain the second most common cause of cancer related death, indicating the some of its cancer cells are not eradicated by current therapies. Sanjal Alam *et al.,* (2013) reported that role of herbal in cancer management and he concluded that some herbs protect the body from cancer by enhancing de-toxic functions of the body.

Use of betel Leafs was known for centuries for its curative properties. In Chinese folk medicine betel leaves are used for the treatment of various disorders and claimed to have detoxification, anti-oxidation, and anti-mutation properties. There are number of research experiments on betel leafs where the leaf extract, fractions, and purified compounds are found to play a role in oral hygiene, and to have various properties including anti-diabetic, cardiovascular, anti-inflammatory/immunomodulatory, anti-ulcer, hepato-protective, anti-infective, etc., Patents were also awarded for some of the biological activities like anti-inflammatory, anti-cancer (Kumar N, *et al.,* 2010). Chewing betel leaves takes a delivery of endeavor and moving parts to salivary gland. It stimulates the release of saliva which is the first step of digestion, as various enzymes in it break down food; create it easy to digest like ginger, figs, fennel etc. Betel leaves has too shown to prevent oral cancer by maintaining the levels of ascorbic acid in the saliva Ascorbic acid is an excellent source of antioxidant, which helps decrease the free radicals in the body, accordingly preventing cancer.

Piper betel leaf show anti-oxidant activity inhibits MCF-7 cell proliferation and increase activities of catalase and super oxide dismutase. Piper betel is a medicinal plant with various biological activities. Anti-proliferative activity of aqueous se extract of piper betel leaf on KB and HeLa cell line , the aim of the study was assessed the effect of the aqueous extract of piper betel plant on the proliferation of cancerous cell lines , that is KB and HeLa cell lines using cytotoxicity assay (Fathilah *et al.,* 2010). Cytotoxic activity of ethanolic extract of piper betel leaf was evaluated using murine (carcinoma and melanoma B-16 cells) cancer cell line by employing MTT assay and Trypan blue dye exclusion method were examined (Roy and vijayalaxmi *et al*.,2013).

In our present study LD- 50 98.4442µl/ml possess good anticancer activity against colon cancer cell lines (HT- 29). Further investigations are needed for identification and purification of the specific anti-cancer components from these plants.

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