

## ANTIPROLIFERATIVE ACTIVITY OF *PIPER BETEL* LEAF EXTRACTS ON HUMAN LUNG CANCER CELL LINE (A549)

DEVJANI BANERJEE\* & BARKHA SHAH

Ashok & Rita Patel Institute of Integrated Study & Research in Biotechnology and Allied Sciences (ARIBAS), New V V Nagar, Gujarat, India.  
Email: devjani.chakraborty@yahoo.com

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### ABSTRACT

**Objective:** *Piper betel* L. commonly known as Paan in India, belongs to family Piperaceae. The leaves are used for treating cough, foul smell in mouth, ozoena, bronchitis, clear throat and stypne. The present study was undertaken to check the anti proliferative activity of *Piper betel* leaf extracts on human lung cancer cell line.

**Method:** The leaves of *Piper betel* was collected from local market of Anand state, Gujarat, India. It was identified and authenticated from Department of Botany, Gujarat University, Ahmedabad, Gujarat, India. Fresh leaves were further processed for extract preparation. The extract was prepared according to WHO Protocol. Lung cancer cell line (A549) was purchased from NCCS Pune and was maintained in media (RPMI1640) supplemented with 10% FBS and incubated in humidified atmosphere of 5% CO<sub>2</sub> and 37°C.

**Results:** Lung cancer cell line (A549) was tested against four *Piper betel* leaf extracts ie; aqueous, methanolic, ethyl acetate and petroleum ether for their anti proliferative activity. IC<sub>50</sub> values represented that maximum anti proliferative activity was found in case of ethyl acetate extract for Lung cancer cell line (A549). Further, the crude extracts were subjected to various chromatographic techniques for the isolation of biomolecules responsible for anti proliferative activity. Eugenol was found to be one, which showed potent anti proliferative activity against Lung cancer cell line (A549).

**Conclusions:** From the present study it was concluded that the leaf extracts of *Piper betel* have anti proliferative and chemo preventive potential and can be use for the treatment of various ailments including human lung cancer.

**Keywords:** *Piper betel*, Leaf extract, Antiproliferative activity, Eugenol, Lung cancer cell line (A549).

### INTRODUCTION

*Piper betel* Linn. belongs to family *Piperaceae*, is a tropical, perennial, dioecious, semi woody plant (creeper) commonly found in Malaysia, Indonesia, India, Srilanka and Philippines and cultivated in many other Asian and East African countries [1]. Betle leaf (*Piper betel*) has many medicinal uses and has been recommended in the ancient scriptures of Ayurveda. The betle leaf is known for its acrid, antiseptic, aphrodisiac, aromatic, astringent, bitter, carminative, hot and stimulant properties [2]. Betle leaf is in use from ancient times as a digestive edible. Applying juice of leaves on wound is a common rural practice. In treatment of gout, arthritis and orchitis betle leaf plays a good role. This herb is also an effective external application for boils. Juice of betle leaves with honey or *gulkand* (rose pedal marmalade) is a good tonic. Studies have shown that betle leaves contain tannins, sugar, carotenes, ascorbic acid and essential oils. Betle essential oils are also generally high in terpenes and phenolic content, with the main constituents being phenol and Eugenol [3, 4].

The rationale behind the present experiment is to check the efficacy of *Piper betel* leaf extracts against anti proliferative activity of lung cancer cell line (A549). A549 is a human lung cancer cell line, it is found to be of epithelial origin and used repeatedly to check anti proliferative activity of various drugs and plant extracts.

Cancer is the uncontrolled growth and spread of cells that can affect almost any part of the body. Lung cancer is one of the five most common cancers prevalent in world for both men and women. More than 11million people are diagnosed with cancer every year. It is estimated that there will be 16 million new cases every year by 2020 [5]. The best possible way to find out the activity of carcinogenic and non-carcinogenic compounds is to do trialing on cell lines. Cell lines play an important role in the cancer biology and are an easy approach to understand the mechanism of carcinogenicity in *in vitro* condition.

### MATERIALS AND METHODS

#### Preparation of leaf extracts

Fresh leaves were collected and dried at room temperature. The extract was prepared according to WHO Protocol CG-06 (1983) [6].

Shade dried pulp was ground with mortar and pestle. Powdered leaves were then packed in soxhlet apparatus and were further processed for extraction. Extracts were prepared in petroleum ether, ethyl acetate, water and methanol. Later on the extracts were properly dried and stored at 4° C in dark bottles. During the study required amount of dried extract was once again re-dissolved in DMSO to get the concentration of our interest. The entire protocol has been mentioned in one of our earlier publication [7]. Various phytochemical tests were carried out to detect the presence of different bioactive components especially flavonoid and total phenol [8]. TLC and HPLC were done to find out the biomolecule present in the leaf extract, responsible for antiproliferative activity.

#### Determination of antiproliferative activity of Human Lung Cancer Cell Line (A549)

Cell lines were derived from NCCS, Pune were free from any kind of bacterial and fungal contamination. Human lung cancer (A549) were cultured in RPMI1640 supplemented with 10% FBS and incubated in humidified atmosphere of 5% CO<sub>2</sub> and 37° C.

#### Plating of cells

Cultured cells were checked for its growth and confluency. After they reached appropriate confluency, media was decanted from flask and trypsin-EDTA was added (2ml). Rounding up of cells was checked under microscope. After cells detached from surface, trypsin was removed carefully, RPMI 1640 supplemented with 10% FBS (2ml) was added and attached cells were scraped off using cell scraper. Cells were then counted and required cells were taken from the flask carefully in order to plate them on 96 well plate (1-3 X 10<sup>6</sup>) cells. Remaining cells were sub-cultured in flasks and kept back in CO<sub>2</sub> incubator [9]. Further the percent viability of the cells was assessed by trypan blue method [10].

#### Microculture Tetrazolium (MTT) assay

This assay is specifically meant to check the % cytotoxicity of a drug or other bioactive compound. It is a colorimetric assay based on the activity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl

thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

As mentioned above, cells were plated approximately  $1-3 \times 10^6$  /plate in a 96 well plate using RPMI-1640 supplemented with 10% FBS. After 24 hours, confluency was checked and at appropriate confluency, drug was added in respective concentrations. After 24hrs of plating, media was decanted from wells and fresh medium

was supplemented with 5% FBS in the wells. Addition of samples, control drug, DMSO was done after 24 hrs according to the above mentioned scheme. After 48 hrs, media was removed carefully and 100  $\mu$ l of DMEM with MTT was added to wells and plate was incubated in CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> for 3hrs. After 3 hrs of incubation, media was once again decanted from the wells carefully and 100  $\mu$ l of 100% DMSO was added to each well and mixed properly in order to make a homogenous solution. 96 well plates were read at 570 nm as well as 490 nm in ELISA reader (Biotek ELX 800) [11] (Table 1).

**Table 1: Scheme for 96 well plate MTT assay**

Untreated	DMSO control (0.5%) 1 $\mu$ l in 200 $\mu$ l							
Positive cont	10ng/ml	100ng/ml	500ng/ml	1000ng/ml	10ng/ml	100ng/ml	500ng/ml	1000ng/ml
Ethyl acetate	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml
Methanol	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml
Water	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml
Pet ether	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml
Eugenol	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml	0.1 $\mu$ g/ml	1 $\mu$ g/ml	10 $\mu$ g/ml	50 $\mu$ g/ml

## RESULTS AND DISCUSSION

The type of *Piper betel* leaf extracts used in the present study has been shown in Table 2. Anti proliferative study was performed with all the four extract followed by DMSO control (used to dissolved extracts) and doxyrubicin (control drug; used in chemotherapy).

**Table 2: Specification of extracts**

Sample No	Solvent system
1.	Ethyl acetate extract
2.	Methanolic extract
3.	Aqueous extract
4.	Petroleum Ether extract
5.	Eugenol

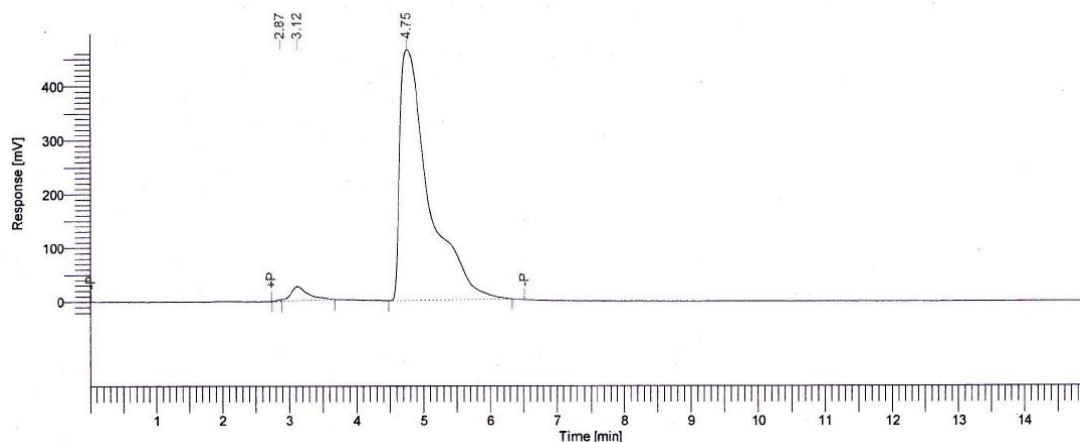
In order to confirm the anti proliferative activity of the *Piper betel* leave extracts on A549 (Human Lung Cancer Cell Line), percent

viable cell was observed by performing trypan blue exclusion technique and further the cytotoxic activity was checked by doing MTT assay. Percent cell survivals of the control cells have been shown below in Table 3 and Fig 2A, which is near about 80-82%.

**Table 3: Characterization of cell line**

Cell Line	Basic characteristic	%Viability
A549	Lung cancer Cell Line (Epithelial origin)	80-81%

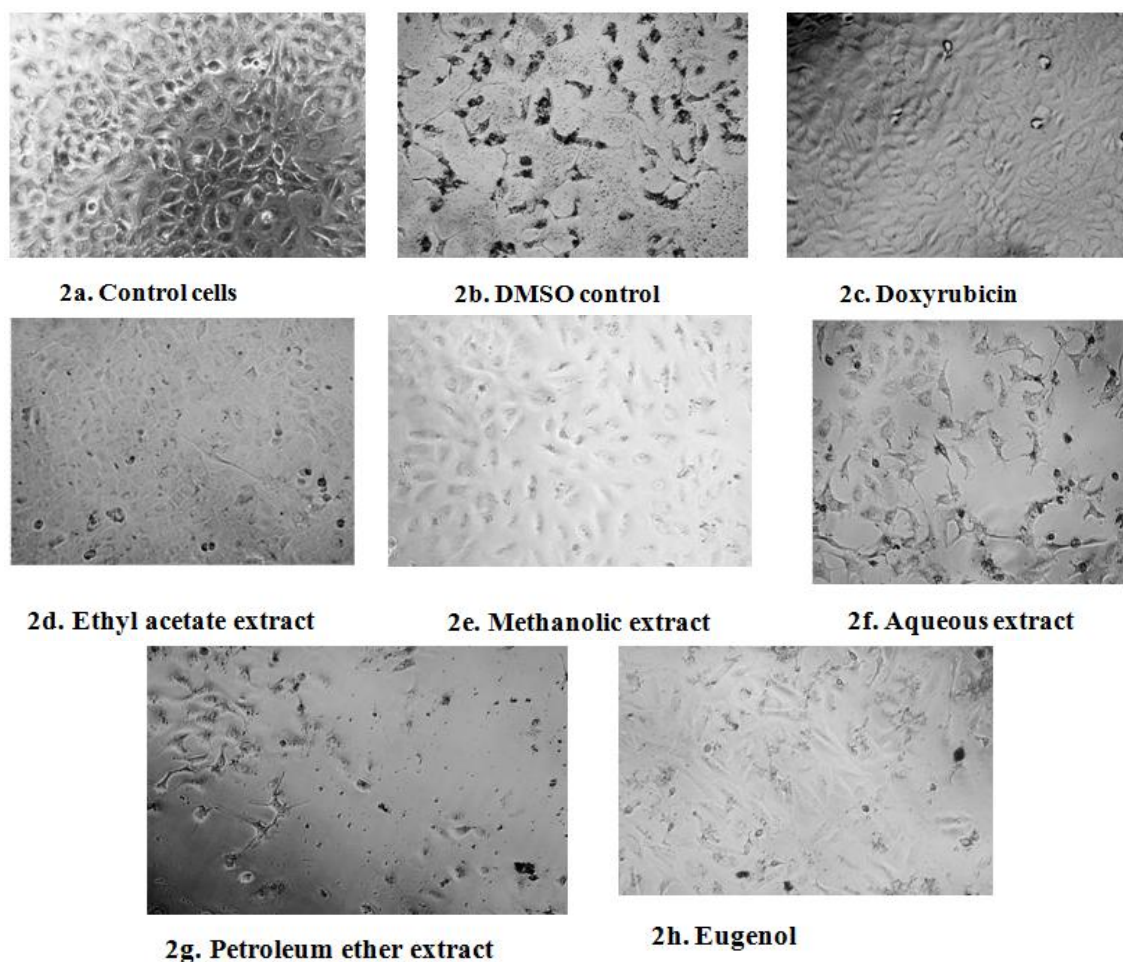
The anti proliferative study was carried out for four different extracts with respect to different concentration as mentioned in table 4 - 8 along with an isolated active compound which was found to be Eugenol (Table 9). TLC and HPLC were performed for the isolation and characterization of Eugenol. HPLC of the isolated Eugenol molecule has been shown below (Fig 1).



**Fig. 2: HPLC of isolated molecule (Eugenol)**

Further, to check the anti proliferative and chemo-preventive ability of the four extracts and Eugenol molecule, IC<sub>50</sub> values were calculated on A549 (Lung Cancer Cell Line), (Table 4 - 9). Percent cell survivals of the control cells along with cells treated with the four extracts were also observed. Doxorubicin was taken as a standard control drug which is normally used as a chemotherapeutic agent against cancer. Below mentioned figures (Fig 2A - 2H), explains the confluency of cells showing cell division and extent of cell proliferation. In control and DMSO control (fig 2A and 2B) the cells are clearly visible, whereas when cells were treated with

control drug doxyrubicin significant percent inhibition was found (Table 4). Fig 2D to 2F shows the cell proliferation in the presence of four different extracts of different concentration (Table 5-8). Maximum inhibition is seen in case of cells treated with ethyl acetate extract whereas minimum inhibition was found to be with methanolic extract. As ethyl extract was showing the significant result, the extract was processed for TLC and HPLC for the separation of Eugenol which has been mentioned above. Fig 2H explains cells treated with Eugenol (Table 9), which also showed significant inhibition in the growth of A549 cell lines.

Fig 1: Response of *Piper betel* Leaf Extracts on A549 Cell LinesTable 4: IC<sub>50</sub> of Standard Doxorubicin drug on A549 cell lines

Std $\mu\text{g/ml}$	% Cell inhibition	IC <sub>50</sub> ( $\mu\text{g/ml}$ )	R <sup>2</sup>
0.01	39.60		
0.1	64.77	0.026	0.999
0.5	81.88		
1	90.32		

Table 5: IC<sub>50</sub> of Ethyl acetate extract of *Piper betel* leaves on A549 cell lines

Std $\mu\text{g/ml}$	% Cell inhibition	IC <sub>50</sub> ( $\mu\text{g/ml}$ )	R <sup>2</sup>
0.1	53.08		
1.0	58.34	0.068	0.719
10	58.36		
50	75.88		

Table 6: IC<sub>50</sub> of Methanolic extract of *Piper betel* leaves on A549 cell lines

Std $\mu\text{g/ml}$	% Cell inhibition	IC <sub>50</sub> ( $\mu\text{g/ml}$ )	R <sup>2</sup>
0.1	12.72		
1.0	17.78	64.6	0.936
10	28.43		
50	41.43		

Table 7: IC<sub>50</sub> of Aqueous extract of *Piper betel* leaves on A549 cell lines

Std $\mu\text{g/ml}$	% Cell inhibition	IC <sub>50</sub> ( $\mu\text{g/ml}$ )	R <sup>2</sup>
0.1	36.15		
1.0	41.94	32.13	0.890
10	43.78		
50	53.38		

Table 8: IC<sub>50</sub> of Petroleum ether extract of *Piper betel* leaves on A549 cell lines

Std µg/ml	% Cell inhibition	IC <sub>50</sub> (µg/ml)	R <sup>2</sup>
0.1	15.02		
1.0	26.50	48.55	0.963
10	28.73		
50	57.53		

Table 9: IC<sub>50</sub> of isolated Eugenol molecule from extract of *Piper betel* leaves on A549 cell lines

Std µg/ml	% Cell inhibition	IC <sub>50</sub> (µg/ml)	R <sup>2</sup>
0.1	49.27		
1.0	50.57	1.31	0.125
10	63.67		
50	72.32		

Various phytochemical tests proved that the leaves extract was also rich in different bioactive molecules like flavonoids and phenols. This has been reported in one of our earlier study [6].

Significant inhibition in the growth of the Lung Cancer Cell Line (A549) was obtained in case of all the extracts as well as isolated Eugenol molecule. Infact, the percent inhibition in case of ethyl acetate extract was found to be more than that of isolated Eugenol molecule. This may be because of the presence of other bioactive molecule having anti proliferative activity in the crude ethyl acetate extract. From the present experiment, it was concluded that the leaf extracts of *Piper betel* shows significant anti proliferative activity on Human Lung cancer cell line (A549) (Fig 2D – 2H). Maximum anti proliferative activity was shown by Petroleum ether extract of *Piper betel* leaves. The anti proliferative activity of *Piper betel* leaf extract on other tissues and its modulation has also been reported earlier [12, 13]. As eugenol was found to be one of the isolated molecule present in the ethyl acetate extract, also showed significant anti proliferative activity. The anti proliferative and anti cancerous activity of Eugenol against various cell lines has been reported earlier. The mechanism, by which Eugenol acts, includes Eugenol-induced cell apoptosis. Where one of the aspects is by activating reactive oxygen radical's generation in the cancerous cells [14].

The intention of the present study was to check the efficacy of piper betel leaf extracts against proliferative activity of A549 cell lines. The early part of the 20<sup>th</sup> century has witnessed an unpredicted evolution of the global pharmaceutical industry. A recent survey conducted by WHO, approximates that 80% of the world population today depend mainly on the traditional medicines for primary health care. Growth is shifting toward emerging markets for traditional plant-based remedies and increasing patronage strengthened by their application as a source of direct therapeutic agents, raw material base for the elaboration of more complex semi-synthetic chemical compounds, models for new synthetic compounds and taxonomic markers for the discovery of new compounds. Considering these aspects we can conclude that ethyl acetate extract of *Piper betel* leaf along with isolated Eugenol molecule can make an allowance for developing chemotherapeutic agent against cancer.

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