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IN-VITRO ANTICANCER POTENTIAL OF CHLOROFORM EXTRACT OF LEAVES OF THULASI VETTILA- A CULTIVAR OF *PIPER BETLE* L. IN KERALA

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ABSTRACT: The present study was designed to evaluate the anticancer potential as well as cytotoxity of Chloroform extract of Thulasivettilacv of *Piper betle* L. is an evergreen perennial creeper belonging to family Piperaceae, and known to possess numerous medicinal properties. Current study focuses on evaluating antiproliferative potentialities of Betel leaves. The antiproliferative effect was tested against two cancer cell lines HeLa and HT 29, while cytotoxity was tested against in a normal myoblast cell line, L6 by MTT assay. Five different concentration of the chloroform extract were used for this study. Qualitative phytochemical analysis of leaf extracts showed the presence of tannins, alkaloids, phenols, flavonoids and glycosides. HeLa cell lines exhibited less antiproliferative activity 25.8 µg/ml ± 0.05% viability at 100µg/ml of extract by MTT assay and the IC_{50} value was found to be 45.19 µg/ml. In case of HT 29 colon cancer cell line, chloroform extract of plant showed 30.32 ± 0.04% µg/ml viability compared with the L6 normal cell line was24.00 ± 0.08% µg/ml. The IC_{50} values were found to be 56.43 µg/ml and 30.26 µg/ml respectively. The extract showed dose dependent anticancer activity. The results revealed that the extract is more potent against HeLa cell lines than HT 29 cell lines.

INTRODUCTION: Cancer is the second cause of death after cardiovascular diseases. The cause of cancer is multifactorial, with hormonal, genetic and environmental factors playing a role in its pathogenesis [1]. Cancer starts with the deformation of a natural cell caused by genetic mutations in DNA. The cost of drugs in use today for the treatment of cancer is too expensive for the majority of the population and therefore the search for some cheap sources of anticancer substances in nature become inevitable.

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Present scenario, various methods are used for cancer treatment such as chemotherapy, but in this method, because of non-selectivity of medicines, a high percentage of healthy cells will be lost with cancer cells. It is one of the most important problem in cancer treatment is damaging natural cells.

Piper betle L. is an evergreen perennial creeper belonging to family Piperaceae and is known to possess numerous medicinal properties. Economically, *Piper* genus is the most important of the family Piperaceae due to its edible purposes and medicinal importance [2]. Betel leaves are not only used directly for chewing purposes but also possesses antioxidant, anti-inflammatory, anti-apoptotic, anticancer and antimicrobial properties [3]. The most important factor determining the aromatic value of the leaf is the amount of particularly the

nature of essential oil present. *Piper betle* leaves from different regions vary in aroma and taste [4]. Hydroxychavicol, an important component of *Piper betle* extract is an allylbenzene class of natural product that has the characteristic odour of *Piper betle* leaves. Hydroxychavicol has been reported to possess anti-mutagenic and anti-carcinogenic activity [5]. The hydroxychavicol inhibits cellular proliferation and induces apoptopsis in pancreatic cancer cells [6]. Current study focuses on evaluating antiproliferative potentialities of Betel leaves.

MATERIALS AND METHODS:

Plant Material: The fresh plant 'Thulasivettila' Cv *Piper betle* L. was collected from Thiruvananthapuram district; Kerala, India. The voucher specimen of the plant [Voucher No. 94684] was prepared and deposited at JNTBGRI Palode for further references.

Preparation of Plant Extract: The leaves of "Thulasivettila" cultivar of *Piper betle* was collected from Thiruvananthapuram district, Kerala, India. The collected plant materials were washed with distilled water and air dried in shade at room temperature. The dried sample was milled in to powder using an electric blender. Dried leaf powder (50g) was serially extracted with Hexane, Chloroform and Methanol, using Soxhlet apparatus. Chloroform extract of Betel leaves was used for the experimental studies.

Phytochemical Screening: The crude extract of plant root was qualitatively tested for the presence of secondary metabolites using standard established methods [7].

Phytochemical Investigation of *Piper betle* Leaves:

Test for Alkaloids (Dragendorff's Method): The extract was warmed with 10 ml of 2% sulphuric acid for 2 min and filtered. A known quantity of aliquot was treated with a few drops of Dragendorff's reagent (glacial acetic acid in a solution of bismuth nitrate and potassium iodide) orange brown precipitate denoted the presence of alkaloids.

Test for Glycosides (Keller - Killani Test): The extract was dissolved in distilled water and added with 2 ml of glacial acetic acid containing one drop of ferric chloride solution followed by 1 ml of concentrated sulphuric acid along the side of the test tube. Brown ring at the interface denoted the presence of glycosides.

Test for Terpenoids (Libermann - Burchard Method): A little of the extract was dissolved in dry chloroform and added three drops of acetic anhydride followed by the addition of two to three drops of concentrated sulphuric acid. Appearance of green colour for steroids while pink colour indicated the presence of terpenoids.

Test for Phenols (Lead Acetate Test): Alcoholic extract was diluted to 5 ml with distilled water and to this few drops of 1% aqueous solution of lead acetate was added. A yellow precipitate was formed, which indicates the presence of phenols.

Test for Tannins (Ferric Chloride Test): To 1-2 ml of aqueous extract, few drops of 5% aqueous $FeCl_3$ solution were added. A bluish-black colour, which disappears in addition of a few ml of H_2SO_4, there is formation of the yellowish-brown precipitate.

Test for Flavonoids (Shinoda Test): The extract was dissolved in methanol and a few pinch of magnesium turnings followed by the addition of concentrated hydrochloric acid drop by drop. Presence of pink colour confirmed the presence of flavonoids.

Biological Screening:

Cell Lines Used: HT-29 colon cancer cell line, HeLa cell line and L6 cell line were obtained [NCCS, Pune]. The cell lines were grown in Dulbecco's modified eagle's media (HIMEDIA) containing 10% fetal bovine serum.

MTT Assay: The MTT assay of the samples was determined according to the method [8]. This assay measures the reduction of yellow 3-(4,5 dimethy-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to form an insoluble formazan precipitated by mitochondrial succinate dehydrogenase only present in viable cells. The cells were washed with 1×PBS and then added 30µl of MTT solution to the culture and incubated at 37 ºC for 3 h. MTT was removed by washing with 1 × PBS and 200µl of DMSO was added to the culture. Incubation was done at room temperature for 30 min. The solution centrifuged for 2 min to precipitate cell debris.

Optical density was read at 540nm using DMSO as blank in ELISA reader. DMSO was used as control without the tested compound. The anticancer drug doxorubicin was used as a positive control in the study.

The percentage cell viability and percentage cell death were calculated with the following formulae:

Percentage viability = (OD of Test / OD of Control) × 100

Cell death % = 1-(OD of sample / OD of control) × 100

RESULTS AND DISCUSSION: Phytochemical analysis conducted on the plant leaf extract revealed the presence constituents such as alkaloids, glycosides, terpenoids, phenols, tannin and flavonoids. From MTT assay, Chloroform extract of leaves showed positive antiproliferative effect against 2 different human cancer cell lines namely of HT-29 (Colon), HeLa (Cervical) and Normal cell line L6 at five different concentrations 6.25, 12.5, 25, 50 and 100µg/ml. Against HT-29cell line leaf extract showed 24.00 ± 0.08% and 25.8 ± 0.05% percentage of viability against HeLa, at 100µg/ml of extract compared with the L6 normal cell line was 30.32 ± 0.04% µg/ml **Table 1**. The IC_{50} values more found to be 30.26 µg/ml and 45.19µg/ml in HT-29 and HeLa respectively. In case of L6 Normal cell line IC_{50} is 56.43µg/ml. The above results confirmed that the cytotoxity of the leaf extract of Thulasivettila cv *Piper betle* L. increased with increase in concentration but the extract showed better activity against HT29 cell lines compared with other HeLa.

Numerous plants have investigated and exhibited to have cytotoxic activity in cancer cell lines. They include that cytotoxic activity of Limonoids from the seeds of *Cipadessa baccifera* against A549, MCF-7, ME-180, HT-29, B-16, ACHN cancer cell lines using MTT assay, and results indicated that compounds cipaferen H, granatumin E and Febrifugin displayed potent cytotoxic activity against B-16, ACHN cell lines [9]. The leaf extract of *Piper betle* extracts antioxidants activity, also inhibits the viability and migration of human breast cancer cells, MCF-7 [10].

TABLE 1: *IN-VITRO* ANTIPROLIFERATIVE EFFECT OF CHLOROFORM LEAF EXTRACT OF THUALSI VETTILA CV *PIPER BETLE* L. BY MTT ASSAY

Sample	Concentration (µg/ml)	Percentage of cell viability		
		HT29	HeLa	L6
Leaf extract	6.25	90.66±0.01	91.39±0.03	90.66±0.01
	12.5	84.00±0.05	75.56±0.07	82.10±0.03
	25	62.66±0.04	67.74±0.04	72.77±0.05
	50	38.66±0.04	44.08±0.09	50.82±0.02
	100	24.00±0.08	25.8±0.05	30.32±0.04
	IC_{50} Values	30.26 (µg/ml)	45.19 (µg/ml)	56.43 (µg/ml)

HT 29 (Colon Cell Line), HeLa: (Human Cervical epithelioid cells), L6 (myoblast cell line) µg/mL: Micrgram per Millilitre, values represent in the results are mean ± SD of three replicates.

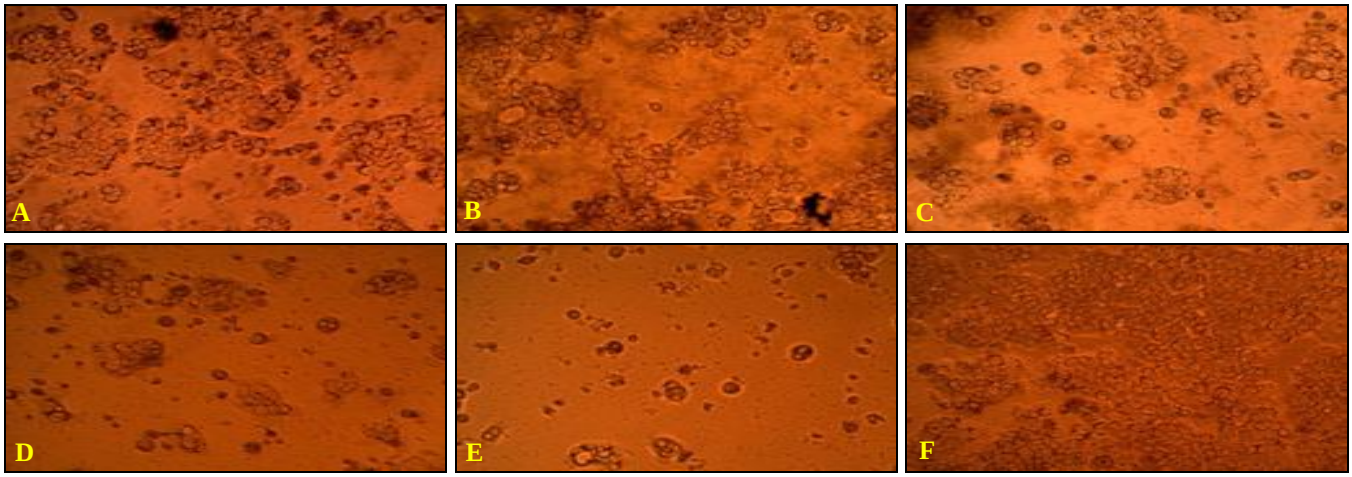

FIG. 1: HT 29 CELL LINES TREATED WITH DIFFERENT CONCENTRATIONS OF CHLOROFORM LEAF EXTRACT
A. 6.25 µg/ml, B. 12.5 µg/ml, C. 25 µg/ml, D. 50 µg/ml, E. 100 µg/ml, F. Control

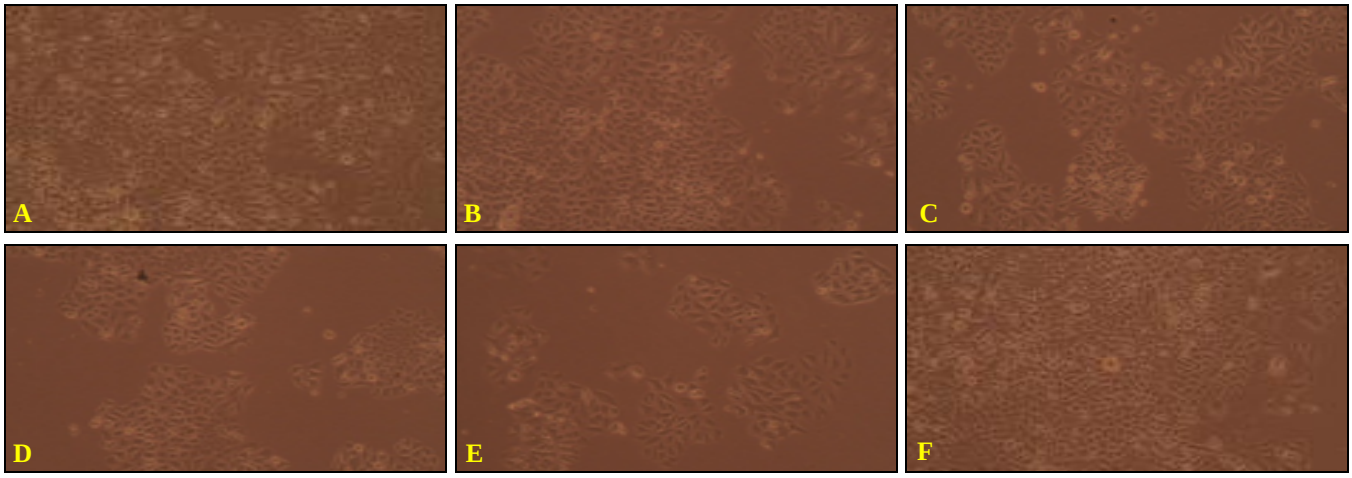

FIG. 2: HELA CELL LINES TREATED WITH DIFFERENT CONCENTRATIONS OF CHLOROFORM LEAF EXTRACT
A. 6.25 µg/ml, B. 12.5 µg/ml, C. 25 µg/ml, D. 50 µg/ml, E. 100 µg/ml, F. Control

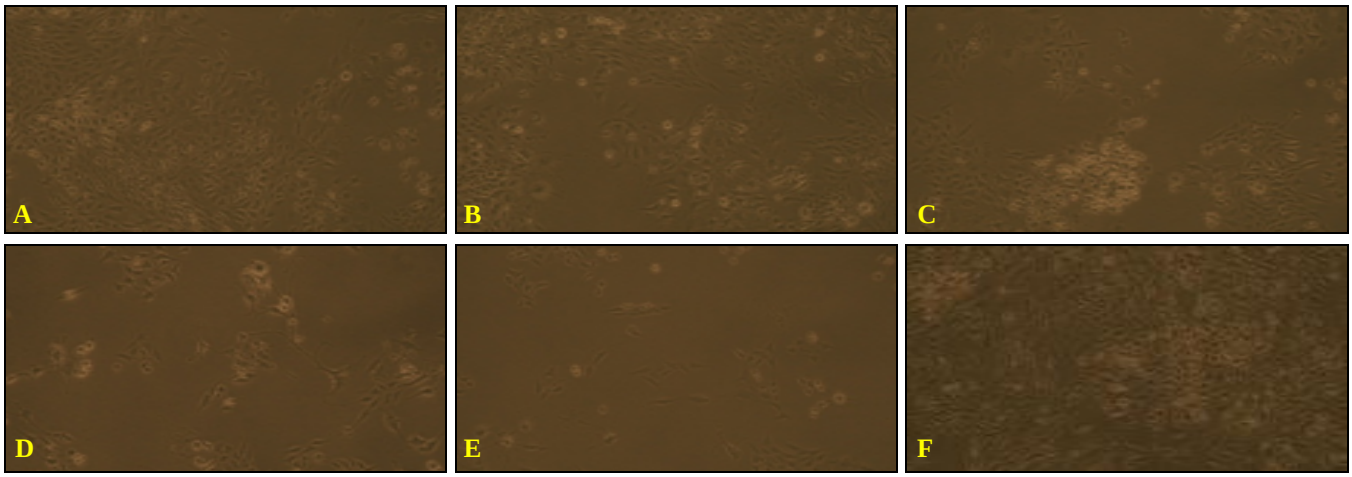

FIG. 3: L6 CELL LINES TREATED WITH DIFFERENT CONCENTRATIONS OF CHLOROFORM LEAF EXTRACT
A. 6.25 µg/ml, B. 12.5 µg/ml, C. 25 µg/ml, D. 50 µg/ml, E. 100 µg/ml, F. Control

CONCLUSION: The result of the present study, the leaf extract showed *in-vitro* antiproliferative activity against the tested human cancer cell lines and less cytotoxity as compared to L6 cell line. Further studies are necessary to elucidate the chemical structure and the active principle of the leaf which is responsible for anticancer activity.

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CONFLICTS OF INTEREST: The author declares no conflict of interest.

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