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FORMULATIONS AND EVALUATION OF BETEL LEAF EXTRACT IN CANCER MANAGEMENT

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ABSTRACT

Chewing betel quid has been linked by several researchers to oral cancer and other potentially harmful conditions of the oral cavity. Conversely, when taken by itself, piper betle leaf may have anti-cancer, anti-helminthic, hepato-protective, and antioxidant properties. In this work, we investigated the anti-cancer properties of aqueous piper betle extract on Hela-cancer cell lines. Piper betle leaf extract was added to the cancer cell lines at escalating concentrations (6.25,12,25,50,100 µg/ml). By looking at the cells under an inverted phase contrast microscope for any discernible changes in cell shape and using the MTT assay method to determine the percentage of viable cells, the cytotoxic effect of the extract on the cells was examined using physical signs of cytotoxic alterations. The MTT assay results demonstrated that the percentage of cancer cells that were viable declined as the extract concentration increased. The highest concentration of 100µg/ml of Piper betle leaf extract resulted in a 43.42% cell viability rate, demonstrating the anticancer activity of the extract. Piper betle leaf's cytotoxic potential could be utilized to create chemotherapeutic drugs, however more in-depth research on the plant's anticancer qualities and the separation of its constituent components are required to demonstrate its value in cancer treatment.

Keywords: Piper Betel, Anticancer activity, Hela cell line, MTT assay.

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INTRODUCTION

The deep green, heart-shaped betel leaf (Piper betle L.) is a part of a vine that climbs roots. It is a profitable crop used for horticulture, medicine, and recreation (Figure.1). In Asian nations, it has long been utilized as a traditional herbal remedy [1]. There are 100 different types of betel leaf (known locally as paan in India), 40 of which are found in India and another 30 in Bangladesh and West Bengal. In addition to Bangladesh, the Philippine Islands, Burma, and other nations, it is extensively grown in several Indian states, including Orissa, Maharashtra, Uttar Pradesh, Tamil Nadu, and Madhya Pradesh [2,3]. Malaysia is the origin place of Paan. Based on its colour, size, taste, and aroma,. It is a perennial climbing vine with a semi-woody stem and short adventitious climbing roots. The leaves are glabrous with an aromatic odor and pungent taste and extensively found in dampforests. A green leafy vine that resembles pepper in growth patterns and can grow as a tiny climber or groundcover. Although the betel leaf plant often grows as an understory groundcover, it is a branching vine that can reach heights of 10 to 15 feet. Generally speaking, it is too delicate to cultivate outside of the tropics. Although it can withstand some drought, the ideal growing environment for plants is warm and humid. The betel leaf is used as a general tonic and in several traditional medicines to treat infections and gastrointestinal problems. As a stimulant, it is frequently chewed alongside betel nut (Areca catechu).

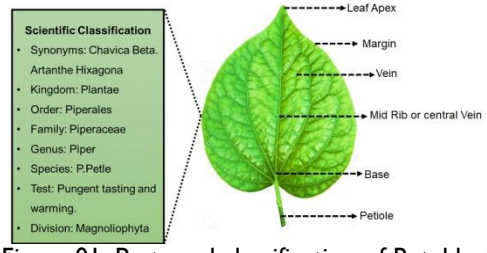

Figure 01: Parts and classification of Betel leaf

Cancer: An Insight

Cancer is a complex and multifaceted group of diseases characterized by the uncontrolled growth and division of abnormal cells within the body. These cells, known as cancer cells, can invade surrounding tissues and, in advanced stages, may spread to other parts of the body through the bloodstream or lymphatic system—a process called metastasis. Phytoconstituents derived from plants have garnered significant attention in cancer research due to their diverse array of bioactive compounds. Phytoconstituents exert their anti-cancer effects through various mechanisms, offering a multi-faceted approach to combatting malignancies. Potential of betel leaf extract in inducing apoptosis (programmed cell death) in cancer cells, inhibiting cell proliferation, and interfering with signaling pathways crucial for cancer progression. Additionally, betel leaf's anti-angiogenic effects and immunomodulatory properties contribute to its potential role in impeding tumor growth.

Types of cancer

i. Breast cancer:

Breast cancer is cancer that develops in breast cells. Typically, the cancer forms in either the lobules or the ducts of the breast.Lobules are the glands that produce milk, and ducts are the pathways that bring the milk from the glands to the nipple. Cancer can also occur in the fatty tissue or the fibrous connective tissue within your breast.

ii. Colon and rectum:

One kind of cancer that begins in the rectum or colon (large intestine) is called colon cancer. The organs that comprise the lower part of your digestive system are your colon and rectum. After excluding some common skin cancers, colon cancer, commonly referred to as colorectal cancer, is the third most prevalent type of cancer in the United States, according to the Centers for Disease Control and Prevention (CDC) Trusted Source. Approximately 1 in 25 women and 1 in 23 men will have colorectal cancer in their lifetime, according to the American Cancer Society (ACS) Trusted Source.

iii. Gallbladder cancer:

Located underneath your liver, the gallbladder is a tiny, sac-like organ that is roughly 3 inches long and 1 inch wide. Its function is to store bile, a substance produced by the liver. Bile is released into your small intestine to aid in food digestion after being stored in your gallbladder. Cancer of the gallbladder is uncommon.

iv. Liver cancer:

Cancer that develops in the liver is known as liver cancer. Your largest internal organ is the liver. It carries out a number of vital tasks to support your body's wound healing, nutrition absorption, and waste removal. The liver is situated directly beneath your ribs in the upper right section of your belly. It is in charge of creating bile, a chemical that aids in the digestion of fats, vitamins, and other nutrients.

v. Kidney cancer:

Each kidney is around the size of a hand and has a bean-like shape. They are situated on either side of your spine in your abdomen. Urine is produced by the kidneys, which also filter waste from your blood. Your kidneys may be affected by a variety of cancer kinds.

vi. Thyroid cancer:

Your body's cells develop out of control when you get cancer. The part of the body where a cancer first appears determines its name. Cancer that starts in the thyroid gland is known as thyroid cancer. The thyroid is a little gland at the base of the throat that resembles a butterfly. It is a component of the body's endocrine system, which creates hormones to control bodily processes.

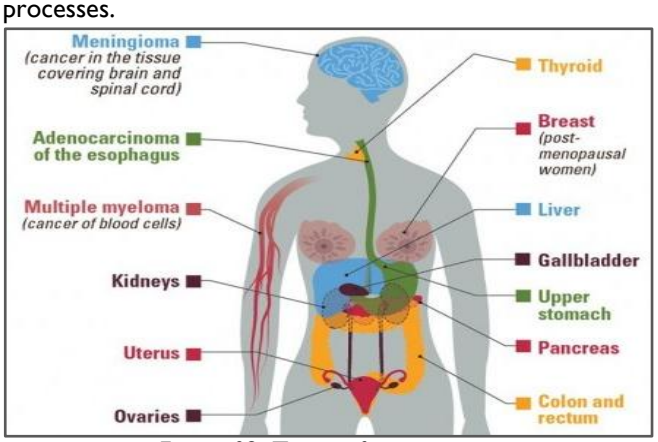

Figure 02: Types of cancer

MECHANISM OF PIPER BETEL LEAVES IN CANCER CELL DEATH.

Piper betel leaf contains a multitude of bio-phenolics such as hydroxychavicol, eugenol, chavicol, and pipers. Convincing data underscore the remarkable chemotherapeutic and chemo-preventive potential of betel leaves against various cancer types. The leaf constituents modulate an extensive array of signaling molecules, such as transcription factors and reactive oxygen species (ROS), to control multiple nodes of various cellular proliferation and death pathways.

Betel leaves contain various antioxidants, such as polyphenols, which may help neutralize free radicals. Free radicals can contribute to cancer development, and by reducing their presence, betel leaf extract may contribute to preventing cancer cell growth. Apoptosis, or programmed cell death, is a natural process that helps eliminate damaged or abnormal cells, including cancer cells. Some studies suggest that betel leaf extract may induce apoptosis in cancer cells, promoting their death. Betel leaf extract has been investigated for its potential to inhibit the uncontrolled proliferation of cancer cells. By disrupting the cell cycle and preventing excessive cell division, betel leaf extract may contribute to slowing down or stopping cancer growth. Betel leaf nutraceuticals target multiple cellular events and play a crucial role in inhibiting tumor growth, and inflammation by decreasing the levels of

cyclooxygenases, COX-1 and COX-2 and stress-inducing events like lipid peroxidation.

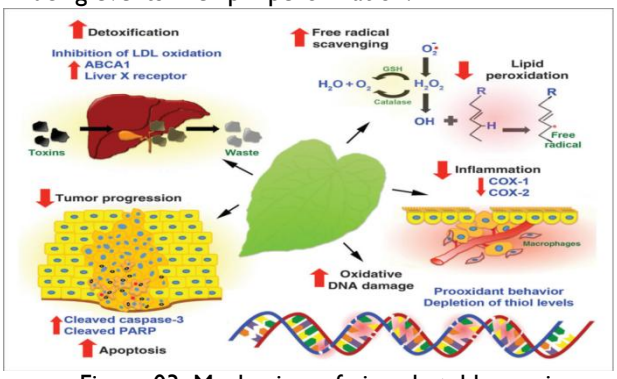

Figure 03: Mechanism of piper betel leaves in cancer cell death

PHYTO PROFILE

- **Drug:** Betel leaf
- **Biological source: -** It is obtained from dried of leaves of Piper betel.
- **Family:** Piperaceae
- **Order:** Piperales
- **Species:** Betle linn
- **Class:** Magnoliapside
- **Morphology**

• Origin- South and Southeast Asia (India, Nepal, Bangladesh, Sri Lanka)
• Popular in Indonesia
• Grow vines height of 15 cm
• Leaves are green and heart shape. length: 5-8cm, width:2-5 cm

Compound constituents: The piper beetle consists of terpinene, P-cymene, carvacrol, chavicol and its substitute, ally catechol, eugenol, chavicol, estragole, oxalic corrosive, malic corrosive, and amino acid. Leaves contain great measured nutrients, especially nicotine corrosive, ascorbic corrosive, and carotin. betel leaf contains several bioactive compounds, including essential oil, tennis, alkaloids, and flavonoids. The basic oil of leaves gives its fragment flavor. β-sitosterol available in roots.

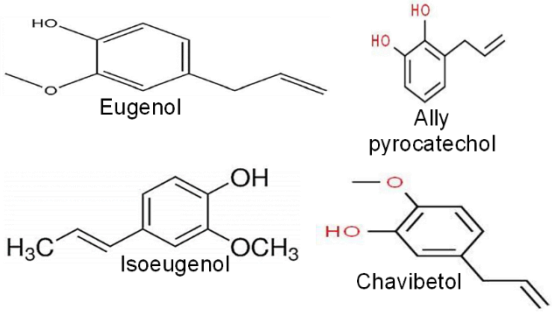

Figure 04: Structure of phytochemical constituents

Uses of betel leaf: Conventional employments of Betel leaves: The utilization of betel leaf can be followed as far back as 2,000 years. Betel leaves help to mend the accompanying ailments. For example,

1. Headache.
2. Scanty or obstructed urination
3. Weakness of nerves.
4. Sore throat.
5. Respiratory disorders.
6. Constipation.
7. Problem of breast milk secretion.
8. Inflammation.
9. Wounds.
10. Boils.

MATERIAL AND METHOD

Table 01: List of materials

Materials	Manufactures
Chloroform	Ad-Nano technologies Private Limited, Karnataka
Sulphuric acid	FDC limited, Chennai
Lead acetate	Central drug house, New Delhi
Phosphate buffer of ph 7.2	Central drug house, New Delhi
Hydroxy chloride	Central drug house, New Delhi
Propylene glycol	Central drug house, New Delhi
Ammonia	Central drug house, New Delhi
Ether	Central drug house, New Delhi

Preparation of Betel leaf extract

After being picked up from the nursery, the cloth was cleaned and allowed to dry in the shade at room temperature before being baked at 20 to 40 degrees Celsius. The dried leaves were placed in a desiccator after being weighed at 100 grams. The Simple Maceration Process was used for the extraction. For seven days, the plant material (100g) was kept at room temperature with periodic shaking after being combined with 1000ml of double-distilled water and 3% chloroform. To get clear liquid extracts, the mixture was filtered using muslin cloth, simple filter paper, and Whatmann filter paper.

RESULTS & DISCUSSION

Preformulation study

A. Chemical test

Chemical test was performed by the ethyl alcohol drug extract of piper betel using standard norms to identify following constituents.

1. Alkaloids: After adding 2 milliliters of Wagner's reagent to 2 milliliters of extract, a brownish precipitate formed, indicating the presence of alkaloids.

2. Cardiac glycosides: Concentrated sulfuric acid was added continually to create a layer after 2 milliliters of extract were dissolved in 2 milliliters of chloroform. The presence of cardiac glycosides is indicated by the deep reddish brown color detected at the steroid ring's interface.

3. Flavonoids: Two milliliters of 10% lead acetate were added to two milliliters of drug extract. Flavonoids are indicated by their yellowish green color.

4. Saponins: 2ml of drug extract was dissolved with 2ml of Benedicts reagent. Blue blackprecipitate reveals the presence of saponins.

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5. Tannins: 2ml of drug extract was treated with 0.1% of ferric chloride. Brownish greenshows the presence of tannin chemical entity.
6. Terpenoids: (Salkowski test) Two milliliters of drug extract were measured, diluted in two milliliters of chloroform, and then carefully mixed with strong sulfuric acid to create a layer. When terpenoids are present, the hue turns reddish-brown.

7. Anthraquinones: In a water bath, 1 milliliter of extract was cooked with 10% HCL for a few minutes. After filtering, it was left to cool. The mixture was heated after an equal amount of CHCl3 was added to the filter and a few drops of 10% ammonia were added. Anthraquinones are present when a rose-pink color forms.

Table 02: Chemical test of herbal betel leaf

Sl.no.	Chemical test	Observation	Results
1	Tannins	Brownish green Color	Positive
2	Anthraquinones	Rose pink color	Positive
3	Flavonoids	Yellowish green color	Positive
4	Alkaloids	Brownish precipitate	Positive
5	Terpenoids	Reddish brown color	Positive
6	Saponins	Blue black precipitate	Positive
7	Cardiac glycosides	Reddish brown color	Positive

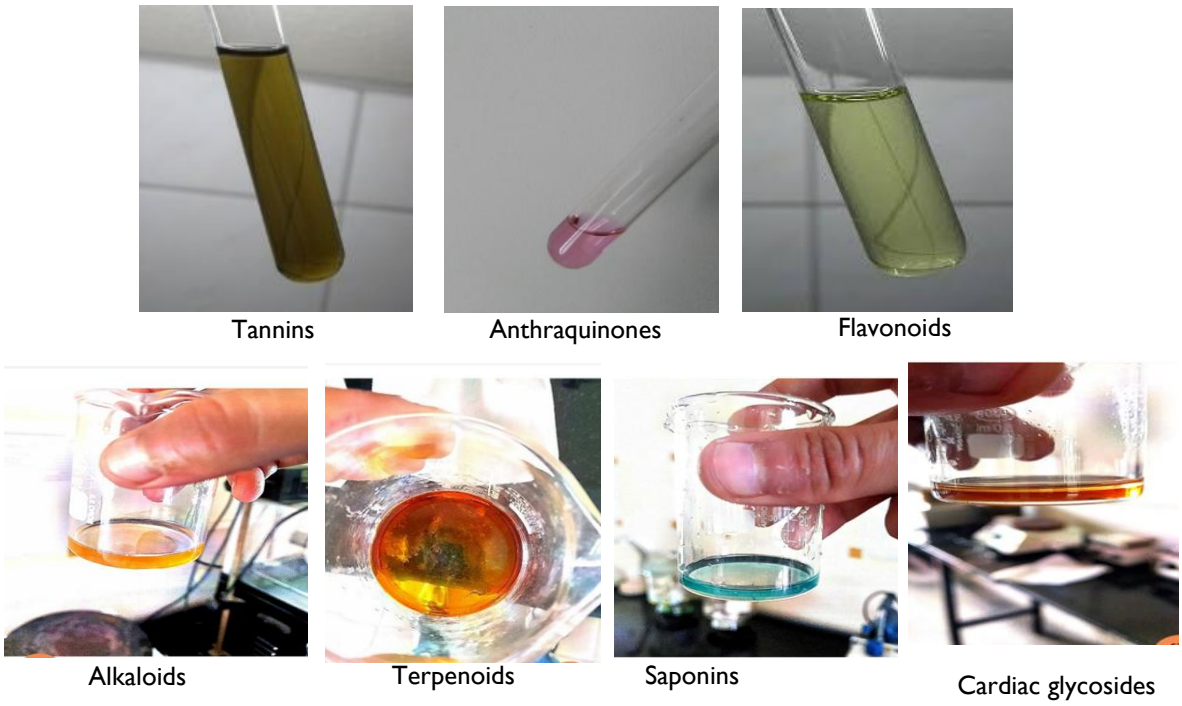

Figure 04: Identification of phytoconstituents of betel leaf

B. Solubility profile

10 ml amount of Distilled water, ethanol, chloroform, propylene glycol was measured andsubjected to determination solubility profile of betel leaf extract

Table 03: Solubility profile of betel leaf extract.

Solvents	Volume ratio (extraction: solvent)	Results
Water	1:1	-
Water	1:2	++
Ethanol	1:1	+++
Chloroform	1:1	+++
Propylene glycol	1:1	+++
Ether	1:1	+++

+++ Miscible ++ Partially miscible – Immiscible

C. Determination of λ_{max} (absorption maxima) of crude extract

λ_{max}Scanning

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To obtain a concentration of 1000µg/mL, 100 mg of crude extract was dissolved in a small amount of pH 7.2 phosphate buffer and then diluted to 100 ml in a volumetric flask. The stock solution was used for this. Different concentrations were to be obtained from different aliquots of stock solution. A UV spectrophotometer was used to scan the resultant solutions for λmax in the 200–400 nm range.

C. Determination of λmax (absorption maxima) of crude extract

Table 04: UV Spectroscopy analysis.

Sl no.	Concentration µg/ml	Absorbance
1	2	0.062
2	4	0.122
3	6	0.165
4	8	0.222
5	10	0.266

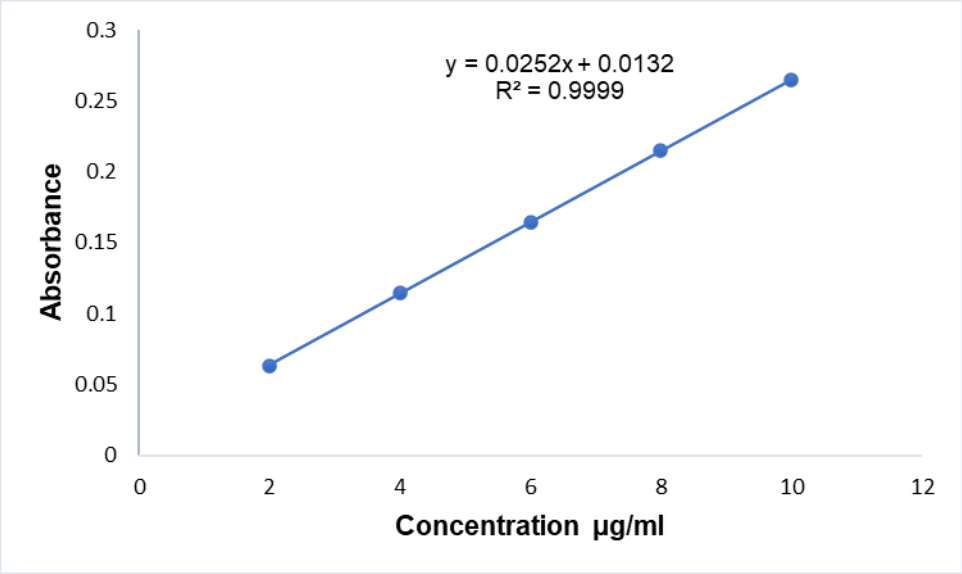

Figure 05: Calibration curve of UV spectroscopy of betel leaf extraction

Preparation of calibration curve

To achieve a final concentration of 20 to 100 µg/mL, aliquots of the PBL extract stock solution (1000 µg/ml) were pipetted out into a series of 10 ml volumetric flasks and diluted with phosphate buffer of pH 7.2. At 280 nm, the absorbance of the resulting solutions was determined. On three days in a row, new solutions were made for the calibration curve.

Cytotoxicity of Piper betleCvThulasivettila

The chloroform leaf extract and essential oil of Piper betle was selected for the in vitro cytotoxicity studies.

Cell lines used and culture conditions

The human cell line for cervical cancer The HeLa cell line was acquired from the National Centre for Cell Science in Pune. It was then kept in Dulbecco's modified Eagle's medium with 10% FBS added, and it was cultivated to confluence at 37°C in 5% CO2 in a humidified environment in a CO2 incubator. After being trypsinized for two minutes, the cells were aseptically transferred to T flasks.

Activity of essential oil on HeLa cancer cell line

HeLa is the oldest and immortal cervical cancer cell lines. It is widely used in cancer research. Doxorubicin is used as standard anticancer compound. In the MTT assay of essential oil of Piper betle L. the percentage of cell viability of HeLa cell line are found to be 84.71%, 74.66%, 47.96%, 37.37% and 24.44% for the sample concentration 6.25µg/ml,12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml respectively (Plate-1). The cytotoxicity of the essential oil on HeLa cell lines is found to be significant as the concentration dependent manner; as the concentration increases, the percentage of inhibition also increases. The IC50 value was found to be 33.61µg/ml for essential oil of Thulasivettila compared with the standard drug Doxorubicin was of 24µg/ml.

Table 05: In-vitro anti-proliferative effect of essential oil on cultured HeLa

Sample concentration (µg/ml)	Percentage cell viability (Essential oil)	Percentage cell inhibition (Essential oil)	Percentage cell viability (Doxorubicin)	Percentage cell inhibition (Doxorubicin))
6.25	84.72±	15.29±	90.45±0.	9.55±0.1

	0.28	0.14	11	5
12.5	74.65± 0.25	25.34± 0.20	63.42±0. 09	36.58±0. 06
25	47.98± 0.22	52.04± 0.11	50.25±0. 06	49.75±0. 09
50	37.38± 0.18	62.63± 0.18	43.87±0. 10	56.13±0. 12
100	24.46 ±0.15	75.56± 0.05	35.56±0. 13	64.44±0. 16

Activity of chloroform leaf extract on HeLa cancer cell line

The decreased cell viability of HeLa cells of about 91.39%, 75.56%, 62.66%, 38.66%, and 24.00% observed at the leaf crude extract concentration of 6.25, 12.5, 25, 50 and 100µg/ml (Figure-9, Plate-1). The IC50 value was found to be 45.19µg/ml for chloroform extract was compared with the standard drug doxorubicin was 24µg/ml.

Table 06: In-vitro anti-proliferative effect of chloroform leaf extract on cultured HeLa.

Sample concentration (µg/ml)	Percentage cell viability (Extract)	Percentage cell inhibition (Extract)	Percentage cell viability (Doxorubicin)	Percentage cell inhibition (Doxorubicin))
6.25	91.39± 0.04	8.6±0.2 0	90.45±0. 11	9.55±0.1 5
12.5	79.56± 0.07	24.44± 0.11	63.42±0. 09	36.58±0. 06
25	67.74± 0.04	32.26± 0.17	50.25±0. 06	49.75±0. 09
50	44.09± 0.09	55.93± 0.04	43.87±0. 10	56.13±0. 12
100	25.8±0. 04	74.20± 0.16	35.56±0. 13	64.44±0. 16

Cell Treatment Procedure

Confluent monolayer cells that were two days old were trypsinized, suspended in 10% growth media, planted on 96-well tissue culture plates, and then incubated at 37°C in an incubator with 5% CO2 that was humidified. The cells were treated with Piper betle leaf extract at escalating concentrations (6.25µg, 12.5µg, 25µg, 50µg, and 100µg) in their respective wells and incubated for 24 hours after the growth media was removed.

Cytotoxicity Assay by Direct Microscopic observation

Using an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera), the entire plate was examined every 24 hours for up to 72 hours. Microscopic observations were captured as photographs. Any discernible alterations in the cells' morphology, such as rounding or shrinking, granulation, or vacuolization in the cytoplasm, were regarded as markers of cytotoxicity.

Cytotoxicity Assay by MTT Assay Method

By using the MTT Assay, the cytotoxicity of the supplied samples on the Hela (purchased from NCCS Pune) cell line was ascertained. Ten thousand cells per well were cultivated in a 96-well plate for twenty-four hours in DMEM media (Dulbecco's Modified Eagle media-AT149-1L) supplemented with 1% antibiotic solution and 10% FBS (Fetal Bovine Serum, HIMEDIA-RM 10432) at 37°C with 5% CO2. Cells were treated with varying quantities the following day (concentration as specified in the excel sheet). Following a 24-hour incubation period, the cell culture was supplemented with MTT Solution (concentration as specified in the excel sheet) and incubated for an additional two hours.

Following the removal of the culture supernatant, the cell layer matrix was dissolved in 100 µl of Dimethyl Sulfoxide (DMSO–SRL-Cat no. 67685), and the results were measured at 540 and 660 nm using an Elisa plate reader (iMark, Biorad, USA). Graph Pad Prism -6 was used to calculate the IC-50. Images were taken with an AmScope digital camera (10 MP Aptima CMOS) under an inverted microscope (Olympus ek2). The following formula was used to determine the percentages of cell viability and cell death:

Percentage of cell viability = (OD of Test/OD of Control) X 100

Percentage of cell death = 100-(OD of sample/ OD of Control) X 100

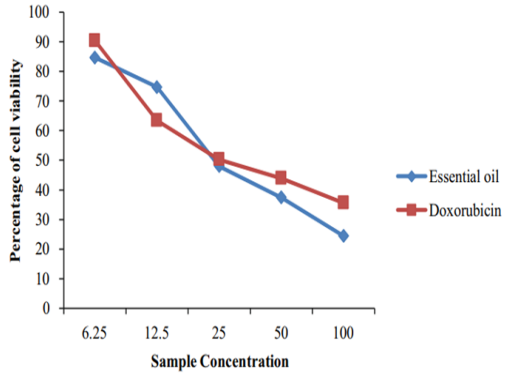

Figure 06: In-vitro proliferative effect on essential oil on cultured Hela cell viability

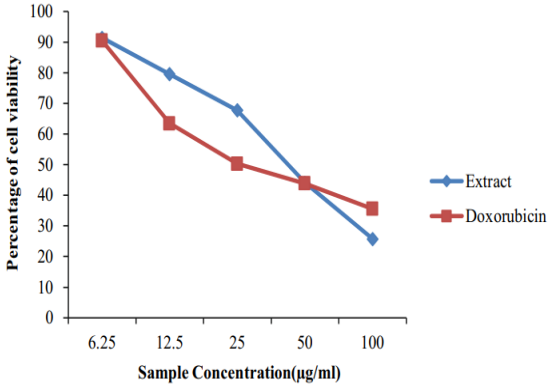

Figure 07: In-vitro proliferative effect on chloroform leaf extract on cultured Hela cell viability

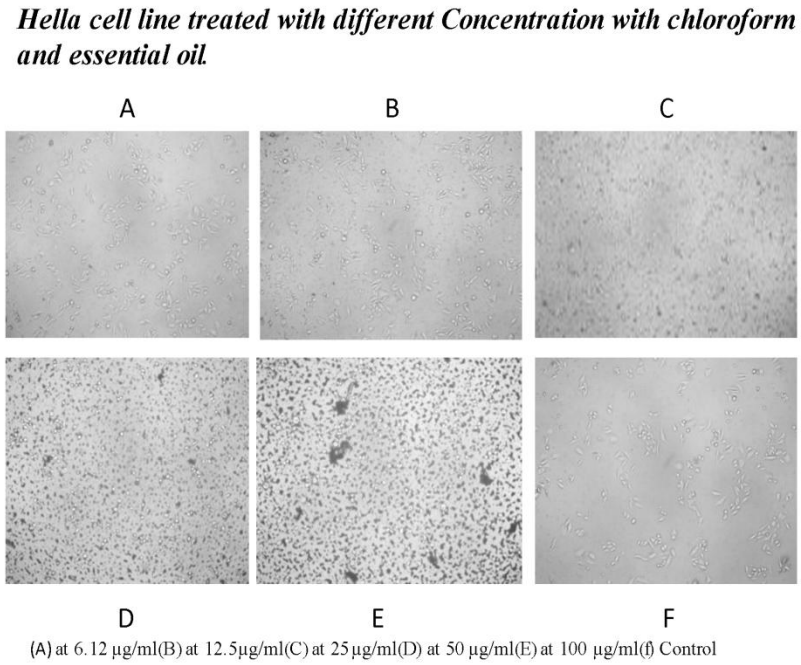

Figure 08: Hela cell line treated with different concentration of Doxorubicin

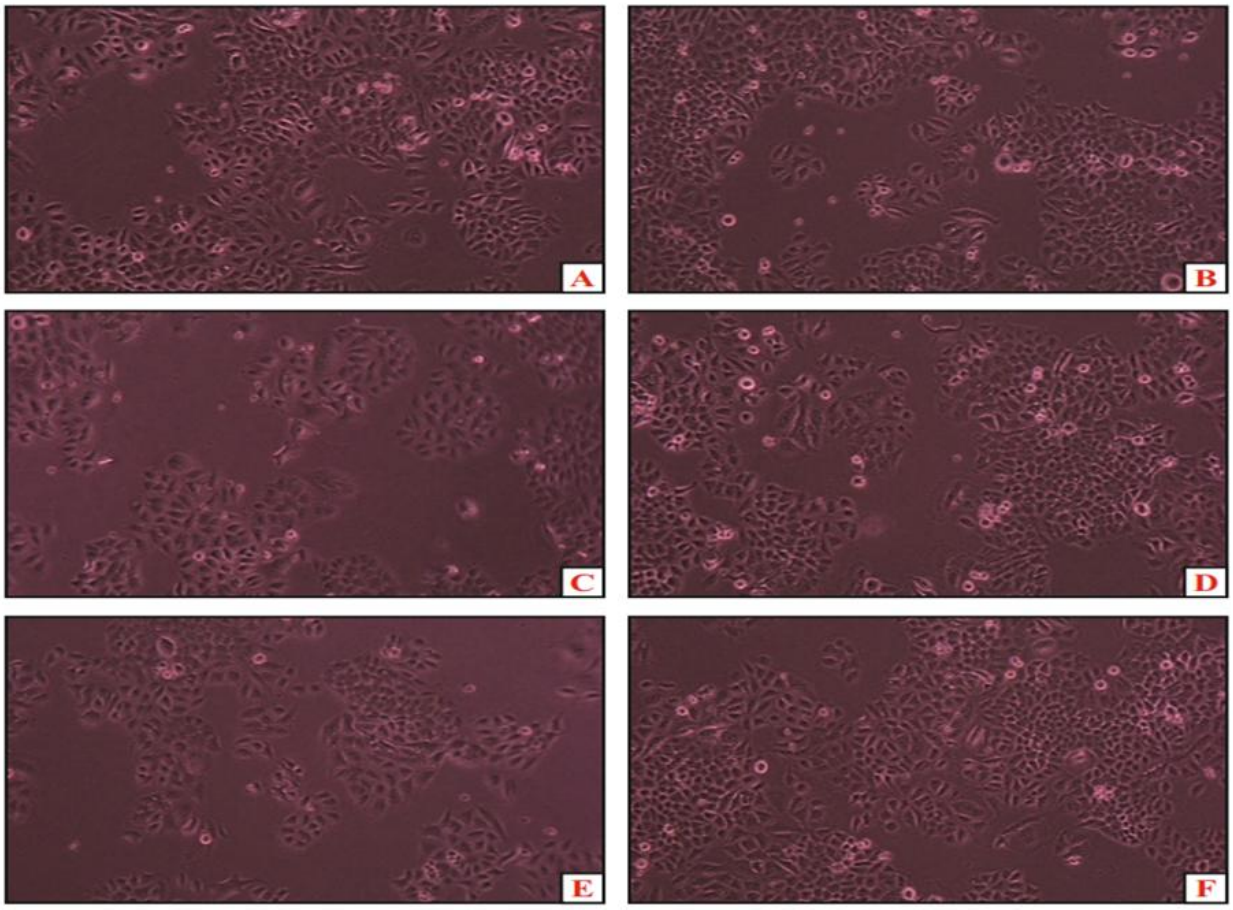

Figure 09: (A) 6.25 µg/ml (B) 12.5µg/ml (C) 25 µg/ml (D) 50 µg/ml (E) 100 µg/ml (F) Control

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MTT Assay:

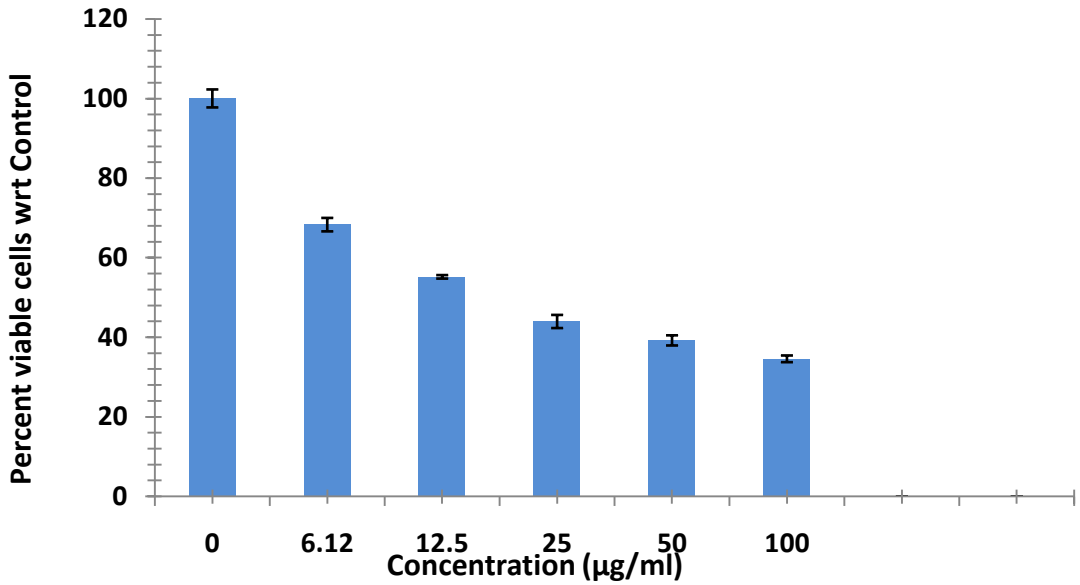

Figure 10: Sample concentration by MTT assay

Table 07: Sample concentration by MTT assay.

Sample Conc.	Test Replicates			
	1	2	3	4
0	0.527	0.491	0.482	0.485
6.12	0.358	0.379	0.387	0.394
12.5	0.326	0.333	0.325	0.332
25	0.304	0.29	0.306	0.327
50	0.2894	0.296	0.287	0.313
100	0.283	0.279	0.297	0.285

DISCUSSION

According to the findings, a leaf extract from the Piper beetle had noteworthy efficacy against cancer cell lines. Under an inverted phase contrast microscope, direct microscopic examinations revealed observable alterations in the cell shape, including cytoplasmic vacuolization, granulation, and rounding or shrinkage of the cells, all of which were thought to be signs of cytotoxicity. We applied increasing amounts of Piper betle leaf extract to the Hela cell line: 6.25µg, 12.5µg, 25µg, 50µg, and 100µg. Under a microscope, we saw cytotoxic alterations that increased in direct proportion to the concentration of Piper betle leaf extract (plate nos. 7, 10, and 13). The MTT technique was used to evaluate the harmful effect of Piper betle leaf extract on Hela cell lines.

Only metabolically active cells can be used for this experiment, which gauges cell viability. A shift in color from purple to a decrease in color graduation indicates that the cells are viable. Optical density (OD) values are used to quantify color intensity, which is directly related to the number of live cells. Our results showed that as the concentration of Piper betle leaf extract increased, the percentage viability of cells dropped (Figure 8, 9). According to the MTT assay results, cytotoxic activity was seen in sample Betel Leaf (IC50 = 48.37 ± 0.22 µg/ml) when the Hela cell line was exposed to varying doses of the sample.

The concentration of an inhibitor, sample, or formulation at which the number of viable cells is cut in half is known as the IC50.

CONCLUSION

Numerous studies, including this one, have demonstrated the strong anti-carcinogenic properties of piper betle leaves. Future research is needed to determine which lead chemicals provide this plant its anticancer and anti-proliferative properties.

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CONFLICTS OF INTERESTS

There are no conflicts of interest.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

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