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Assessment of Phytochemicals, Antioxidant and Anticancer Potential of Ethanolic Leaf Extracts of *Plumbago zeylanica*

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Abstract: *Plumbago zeylanica* L., a medicinal plant traditionally used in Ayurveda, mainly for its roots, while the therapeutic potential of its leaves remains less studied. This study aimed to evaluate the phytochemical composition, antioxidant activity, and anticancer effects of *P. zeylanica* ethanolic leaf extracts. Phytochemical analysis and antioxidant activity was studied using standard methods. Anticancer activity against HeLa cells was evaluated using MTT, Scratch, Clonogenic, Methylene blue, and Hoechst staining assays. The ethanolic extract showed higher flavonoid content (26.95 ± 0.746 mg QE/g), FRAP activity (26.23 ± 0.891 mg AAE/g), and reducing antioxidant power (19.39 ± 1.16 mg AAE/g). The radical scavenging activity was significantly higher for ABTS assay (75.1%) as compared to DPPH assay (26.8%) and superoxide scavenging assay (18.88%). Although the extract exhibited marginal cytotoxicity against HeLa cells, it induced significant morphological alterations including cell rounding at 200 µg/mL. Cell migration and colony formation was abolished by 50 µg/mL extract. Staining assays indicated increased cytoplasmic granularity, compromised membrane integrity and modest apoptosis at 200 µg/mL. Overall, the findings demonstrate that the ethanolic leaf extract of *P. zeylanica* possesses moderate anticancer potential characterized primarily by anti-proliferative and anti-migratory activities, warranting further investigation of its phytochemicals as potential chemopreventive or chemosensitizing agents.

Keywords: *Plumbago zeylanica*, Ethanolic extract, Antioxidant assays, HeLa cells, anticancer activity

1. Introduction

Alcohol Cancer disease occurs due to abnormal cell proliferation, additionally genetic complexity, heterogeneity, and ability to develop resistance to conventional therapies poses a major challenge to develop effective treatment for malignant tumors. The lack of specificity of conventional chemotherapeutic drugs results in damage to both tumor cells and normal healthy tissues, often contributing to various adverse effects viz. pulmonary toxicity, cancer-related fatigue, peripheral neuropathy, cardiotoxicity, and cognitive, gastrointestinal, and musculoskeletal complications (Nekhlyudov et al., 2022).

Despite development of varied novel targeted therapies for cancer, plant-based medicines have always been regarded as promising, a relatively safer and cost-effective treatment strategy. Natural products are receiving more attention as anticancer agents, due to their potent pharmacological activity at low concentrations, minimal toxicity to normal cells and potential to overcome drug resistance, thus can lessen side effects and improve the efficacy of cancer treatments (Gostner et al., 2012). Given the heterogeneous nature of cancer and the involvement of multiple molecular targets and oncogenic pathways, monotherapy often demonstrates limited efficacy in both the treatment and prevention of cancer. Most of the solid tumors viz triple-negative breast cancer, pancreatic ductal adenocarcinoma, glioblastoma multiforme, non-small cell lung cancer, and ovarian cancer pose particular therapeutic challenges due to their complex biology, pronounced heterogeneity, aggressive progression, and high propensity for drug resistance (Bhatia et al., 2020). Therefore, to treat such cancers, combination therapy is frequently employed which comprises of using multiple drugs or plant extracts combined with chemotherapy or a combination of one or more natural compounds which show higher thera-peutic effectiveness (Lin et al., 2020; Talib et al., 2022; Wang et al., 2023).

Plumbago zeylanica L., is a medicinal plant that belongs to the

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family Plumbaginaceae. This perennial herbaceous plant is commonly known by different names; as Chitraka/Chitramol in Sanskrit, White Leadwort/Ceylon leadwort/Doctorbush in English, Sheetraj in Hindi (Ajayi et al., 2019; Jain et al., 2020). Occurrence of plant is reported in tropical and subtropical areas worldwide. In India, this plant is widely distributed in north-east India in the wilds of Assam and Meghalaya (Ravindran et al., 2017), Uttar Pradesh, Andhra Pradesh, Karnataka, Kerala and Bengal (Dev et al., 2018). *P. zeylanica* L. has been traditionally valued for its extensive medicinal properties, with different plant parts used for diverse therapeutic purposes over centuries. The root and root bark are particularly significant and are widely incorporated into various Ayurvedic formulations. The roots possess laxative, expectorant, tonic, abortifacient, and appetite-stimulating activities, and are beneficial in the management of rheumatism, laryngitis, scabies, and spleen disorders (Chauhan Mukul, 2014; Roy Arpita & Bharadwaja, 2017). They are also traditionally employed for correcting chronic menstrual irregularities, treating viral warts and chronic neurological conditions, serving as anticancer agents, and acting as rejuvenators in conditions like obesity. Additionally, roots are used for digestive ailments including loss of appetite, indigestion, piles, worm infestations, colitis, ascites, and liver disorders (Chauhan & Chauhan, n.d.). The leaves of *P. zeylanica* are utilized in the treatment of scabies, sores, and swellings, and are considered effective against dysentery. Leaf paste is commonly applied topically to alleviate painful rheumatic conditions and chronic, itchy skin disorders (Vishnukanta & Rana, 2010). A decoction prepared from the seeds is traditionally used to reduce muscular pain.

Phytochemical investigations of *P. zeylanica* L. have revealed the presence of a diverse array of bioactive constituents. Preliminary screenings confirm the occurrence of alkaloids, carbohydrates, triterpenoids, flavonoids, gums, mucilage, proteins, fatty acids, and saponins. In addition, multiple classes of secondary metabolites including naphthoquinones, flavonoids, alkaloids, glycosides, saponins, steroids, tannins, triterpenoids, coumarins, carbohydrates, phenolic compounds, fixed oils, fats, and proteins have been reported in this species (Chauhan Mukul, 2014; Vishnukanta & Rana, 2010).

Of all the chemical constituents, plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) is the principal active compound primarily present in roots in higher amounts with only about 1% in the whole plant (Dohare Bharti et al., 2015; Priya Velammal et al., 2016). Despite its long-standing traditional use and emerging scientific evidence, *P. zeylanica* leaves remains insufficiently investigated, particularly with respect to leaf bioactives correlating with functional antioxidant and anticancer activities.

Thus our present research focuses on assessing the phytochemical and antioxidant potential of *P. zeylanica* ethanolic leaf extract and investigating its antiproliferative and anticancer activity against HeLa cervical cancer cells.

2. Materials and methods

2.1 Collection of Plant material and Preparation of Extracts

P. zeylanica plants were collected from the Western Ghats, Satara District, Maharashtra, India, and were identified based on their morphological characteristics by Dr. Nimbalkar, Assistant Professor, Shivaji University, Kolhapur, Maharashtra. No voucher specimen was deposited in a recognized herbarium; therefore, a voucher specimen number is not available. Plant material was washed, air-dried at room temperature and ground into a fine powder in a mortar and pestle using liquid nitrogen.

For extract preparation by maceration method, 5 g leaf powder and 60 mL of ethanol was mixed in a glass jar and incubated on a shaker for 24 h. The mixture was centrifuged at 10,000 rpm for 10 min, the supernatant was collected and the leaf residue was re-extracted twice using fresh ethanol. All the filtrates were combined together yielding a 5% (w/v) stock, which was diluted to working concentrations of 0.1%, 0.5% and 1% and used for phytochemical analysis.

For Cell based assays, 10 g leaf powder was subjected to Soxhlet extraction using absolute ethanol. The extract obtained was concentrated by rotary evaporator, air dried and stored at -20°C. The dried extract was reconstituted in dimethyl sulfoxide (DMSO), filter-sterilized and diluted in sterile DMSO to prepare working stocks of 0.1, 1, 10, 100, and 200 mg/mL. All the extracts were stored at -20 °C until further use. The percentage yield of ethanolic extract was 10.84%.

2.2 Phytochemicals analysis of *Plumbago zeylanica* extract

2.2.1 Estimation of Total Phenolic content

Briefly, 1 mL of the extract (0.1%) was mixed with 200 µL of Folin–Ciocalteu reagent and incubated in the dark for 6 min. Subsequently, 2 mL of 7% sodium carbonate solution was added, and the mixture was further incubated in the dark for 90 min. The blue-colored complex formed was measured at 760 nm. Gallic acid was used as the standard and the total phenolic content was expressed as milligram equivalence of gallic acid per g of plant extract (mg GAE/g) (Singleton & Rossi, 1965).

2.2.2 Estimation of Total Flavonoid content

For the assay, 1.0 mL of extract (0.1%) was mixed with 1.5 mL of 2% methanolic aluminium chloride ($AlCl_3$) and incubated in the dark for 10 minutes. The intensity of yellow-coloured complex formed was measured at 368 nm. Quercetin was used as standard and flavonoid content was expressed as milligram equivalence of quercetin per g of plant extract (mg QE/g) (Luximon-Ramma et al., 2002).

2.3. Assessment of antioxidant activity of *Plumbago zeylanica* extract

2.3.1 DPPH Radical Scavenging Assay

1 mL of extract (1%) and 1 mL of 0.3 mM DPPH in methanol was mixed and incubated in the dark for 30 minutes. The absorbance was measured at 517 nm. Ascorbic acid was used as standard (Medpilwar et al., 2015). The percent radical scavenging activity was calculated as:

% Radical scavenging activity:

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

2.3.2 ABTS Radical Scavenging Assay

For the assay, ABTS radical cation ($ABTS^{+}\cdot$) was generated by mixing equal volumes of 7 mM ABTS solution in methanol and 2.45 mM potassium persulfate solution and incubated in the dark for 16 hr. This $ABTS^{+}\cdot$ stock solution (blue-green color) was diluted with methanol till an absorbance of 0.70 ± 0.02 at 734 nm. Then 1 mL extract (0.5%) and 2.5 mL of the freshly diluted ABTS solution was mixed gently and the absorbance was recorded at 734 nm at 0 min and after 4 min of incubation at room temperature. Ascorbic acid solution (1 mg/mL) was used as standard (Pai et al., 2015; Ghatak et al., 2015).

The percent scavenging activity was calculated using the expression % Radical scavenging activity:

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

2.3.3 FRAP assay

FRAP assay was done as described by (Benzie & Strain, 1996) with slight modification. A total of 100 µL extract (30µL of 1% extract + 70µL of distilled water) was mixed with 900 µL of the freshly prepared FRAP reagent (25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) prepared in 40 mM HCl, and 2.5 mL of 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$). The resulting mixture was vortexed briefly and incubated at room temperature. Absorbance was recorded at 593 nm at 0 min and 4 min of incubation to monitor the reduction of Fe^{3+}-TPTZ to the ferrous complex (Fe^{2+}-TPTZ), forms an intense blue colour. Ascorbic acid served as standard and FRAP reducing activity of extract was expressed as milligram equivalence of ascorbic acid per g of plant extract (mg AAE/g).

2.3.4 Superoxide anion radical scavenging (SO) assay

The superoxide anion radical scavenging was measured as described by (Robak & Gryglewski, 1988) with slight modifications. To reaction mixtures comprising of 0.5 mL NBT solution (0.3 mM), 0.5 mL NADH solution (0.936 mM), and 0.5 mL Tris-HCl buffer (16 mM, pH 8.0), 0.5 mL of the gallic acid standards and *P. zeylanica* extract (0.5%) were added. The reaction was initiated by addition of 0.5 mL of phenazine methosulfate (PMS, 0.12 mM), which generates superoxide radicals in the presence of NADH. On incubation at 25°C for 5 minutes, the superoxide radicals subsequent reduce NBT to formazan which was measured at 560 nm.

$$\% \text{ inhibition} : \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

2.3.5 Reducing power assay

The reducing power of the ethanolic extract was determined by method of (Athukorala et al., 2006). 1 mL of the extract was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 30 mM potassium ferricyanide and incubated at 50°C for 20 minutes. Then 2.5 mL of 10% trichloroacetic acid (TCA) was added to the reaction mixture, centrifuged at 3000 rpm for 10 minutes. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 6 mM ferric chloride solution and absorbance was measured at 700 nm. Reducing power was expressed in terms of standard ascorbic acid equivalent (mg AAE/g).

2..4. Anticancer study

2.4.1 Cell culture

HeLa cells were procured from NCCS, Pune and cultured in Dulbecco's Modified medium (Cell clone) supplemented with 10% heat-inactivated fetal bovine serum (Cell clone), Penicillin-Streptomycin mix (100 U/mL penicillin and 100 µg/mL Streptomycin). Cells were incubated at 37°C in a humidified atmosphere with 5% CO2, cell feeding with fresh media was done on alternate days based on cell density. Upon 70% confluency, cells were trypsinized, counted and seeded for assays, passaged or freezed down.

2.4.2 MTT cytotoxicity assay

Briefly, a total of 3000 cells/200 µL were seeded per well of a 96-well plate and 24 h after seeding these were treated with various concentrations of *P. zeylanica* extracts for 48 hours at 37°C. Media control and DMSO control were maintained for all experimental sets. All the test and control samples were set as quadruplicate. Post treatment, 40 µL of MTT solution (5 mg/ mL in 1 X PBS) was added to each well and incubated for 4 h. The formazon crystals formed were dissolved in 100 µL of DMSO and absorbance was measured on ELISA plate reader at 540 nm. The cell viability was expressed as a percentage of the absorbance in control cultures (Mosmann, 1983).

2.4.3 Scratch assay

The migratory potential of cells treated with *P. zeylanica* extract was assessed by scratch assay. Briefly 4×10^4 cells/200 µL were seeded per well in a 96-well plate and incubated for 24 hours. In the confluent monolayer formed, a scratch was made using a sterile micropipette tip. The media from each well was carefully aspirated and replaced with 200 µL of fresh medium containing 25 µg/mL and 50 µg/mL of extracts. Cell migration into the wound area was monitored and photomicrographed over a period of 24 and 48 hours.

2.4.4 Clonogenic assay

The colony forming ability of cells was assessed by clonogenic assay. Briefly, 500 cells per well were seeded into 6-well plates and allowed to attach for 24 hours. The cells were subsequently treated with *P. zeylanica* extract at concentrations of 25 µg/mL and 50 µg/ mL for 24 hours. Following treatment, the medium was replaced with fresh extract-free medium. Colony formation in both control and treated groups was then monitored for 9 days.

2.4.5 Methylene blue assay

The cytological staining and quantification of dye retention in cells treated with *P. zeylanica* extract was studied by modified methylene blue assay as described by (Felice et al., 2009). For the assay, 1.7 X105 cells were seeded in 24-well plate and allowed to adhere for 24 hours. Then the cells were treated with varying concentration (1–200 µg/mL) of the extract for 48 hours. Post-treatment, the medium was carefully aspirated, cells were gently rinsed with 1X PBS and subsequently fixed and stained with methylene blue solution (0.6% methylene blue and 1.25% glutaraldehyde in Hank's Balanced Salt Solution (HBSS) for 60 min at 37 °C. Excess dye was discarded followed by six washes with distilled water. The wells were then air-dried and the dye retained in the cells was eluted using elution buffer (50% ethanol, 49% PBS, 1% acetic acid). The eluted dye was collected in microfuge tube, centrifuged at 1500 rpm for 3 min, the resulting supernatant was transferred to a 96-well plate and quantitated by measuring the absorbance at 630 nm using a microplate reader.

2.4.6 Hoechst staining

To determine the apoptotic cell population, Hoechst single staining was performed. 8X103 cells/well were seeded in 96-well plate and incubated for 24 hours, before drug exposure. 24 hours post drug treatment, single staining (Hoechst) was performed. Images were captured at 20X in DAPI filter in Cytation 5. This assay was performed at Lifesenz labs.

2.5 Statistical Analysis

All the assays were performed in triplicate and results were expressed as mean ± S.D. Statistical analysis of phytochemical and antioxidant assay was done on Graphpad (version 9.4.0) software. Data was analyzed using One way Analysis of variance technique (ANOVA) test and multiple comparisons was done by Tukey's test,

and *p<0.05, **p<0.01, and ****p<0.001 were considered statistically significant.

3. Results

3.1 Phytochemical Analysis and Antioxidant activity

Phytochemical analysis of *P. zeylanica* ethanolic extract was done using standard assays. The total flavonoid content, FRAP activity, reducing power and total phenolic content of leaf extract was reported to be 26.95± 0.746 mg QE/g, 26.23± 0.891 mg AAE/g, 19.39 ± 1.16 mg AAE/g and 6.38± 0.375 mg QE/g respectively. The statistical analysis by Tukey's multiple comparison test showed that the total flavonoids, FRAP and reducing power activity of the extract was significantly high as compared to total phenolics (**Fig 1**).

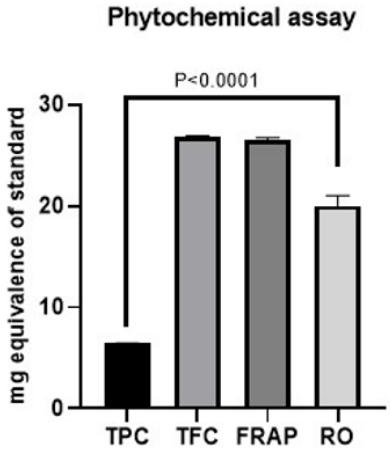

Fig. 1. Phytochemical assays of *Plumbago zeylanica* leaf ethanolic extract. Values are expressed as milligram equivalence of standard antioxidant per gram of plant extract. The total flavonoid content, FRAP activity and reducing power of ethanolic extract was significantly higher as compared to total phenol content (p<0.0001).

The antioxidant activity, expressed as percent radical scavenging, was 26.8%, 18.88%, and 75.1% for the DPPH, superoxide anion (SO), and ABTS assays, respectively. Statistical analysis using Tukey's multiple comparison test demonstrated that the radical scavenging activity measured by the ABTS assay was significantly higher than that measured by both the DPPH and SO assays (p < 0.0001). Furthermore, comparison between the DPPH and SO assays revealed that the DPPH assay exhibited significantly greater radical scavenging activity than the SO assay (p = 0.0009) (**Fig. 2**).

3.2 MTT Viability Assay

The cytotoxic potential of the ethanolic extracts of *P. zeylanica* on HeLa cells was evaluated using the MTT viability assay. The results showed a marginal decrease in cell viability compared with the untreated control following 48 h of extract treatment (Fig. 3). However, morphological analysis revealed distinct cellular alterations, with cells exhibiting a striated appearance and rounding at a concentration of 200 µg/mL (**Fig. 4**).

3.3. Scratch Assay

The effect of *P. zeylanica* extract on cell proliferation and migration was assessed using a scratch assay. Control and DMSO-treated HeLa cells exhibited significant proliferation and migration, resulting in substantial wound closure after 48 h. Treatment with 25 µg/mL of *P. zeylanica* extract resulted in moderate migration of cells in the wounded area, whereas cells treated with 50 µg/mL extract showed a marked reduction in migration, with sparse cell distribution along the wound margins (**Fig 5**). These findings indicate that *P. zeylanica* extract inhibits HeLa cell proliferation and migration in a concentration-dependent manner.

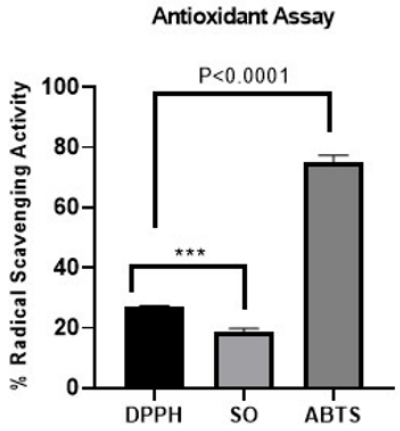

Fig. 2. Antioxidant activity of *Plumbago zeylanica* leaf ethanolic extract determined by DPPH, superoxide anion (SO), and ABTS radical scavenging assays. The ABTS assay exhibited significantly higher percent radical scavenging activity than both the DPPH and SO assays (p<0.0001). Furthermore, the DPPH assay showed significantly greater radical scavenging activity than the SO assay (p = 0.0009). Data were analyzed using Tukey's multiple comparison test

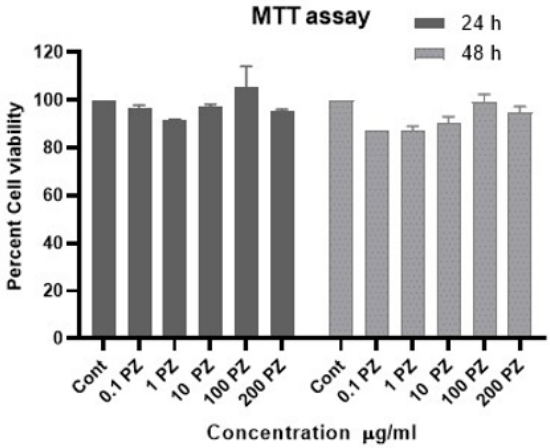

Fig 3. Effect of *Plumbago zeylanica* leaf ethanolic extract on HeLa cell viability assessed by the MTT assay. HeLa cells were treated with the ethanolic extract of *Plumbago zeylanica* (PZ) for 24 and 48 h, and cell viability was determined using the MTT assay. Cell viability is expressed relative to the untreated control (Cont). Treatment with the extract resulted in a marginal reduction in cell viability at both time points.

3.4 Clonogenic Assay

In the clonogenic assay, control and DMSO-treated HeLa cells demonstrated a progressive increase in colony size over a period of 9 days, indicating normal proliferative capacity. In contrast, HeLa

cells treated with 25 µg/mL and 50 µg/mL of *P. zeylanica* extract exhibited scattered and reduced colony formation, suggesting that the extract exerts an inhibitory effect on the clonogenic potential of HeLa cells (**Fig. 6**).

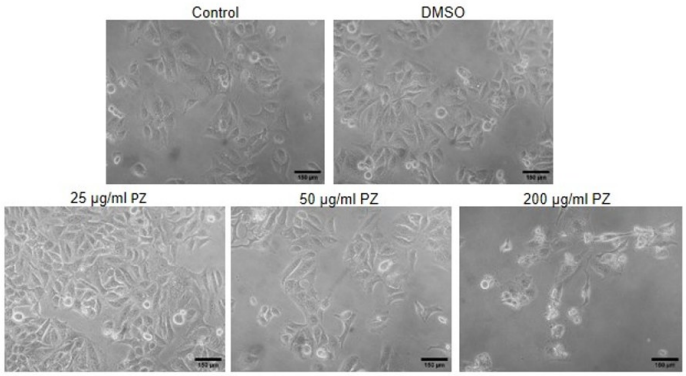

Fig. 4. *Plumbago zeylanica* leaf ethanolic extract induces alterations in morphology of HeLa cells. Representative brightfield micrographs of HeLa cells treated with 25 µg/mL, 50 µg/mL and 200 µg/mL of *Plumbago zeylanica* (PZ) ethanolic extract (200X magnification, scale bar 150 µm). Cell population decreased with increasing concentration of ethanolic extracts and morphology appeared striated and rounded evidently at 200 µg/mL concentration.

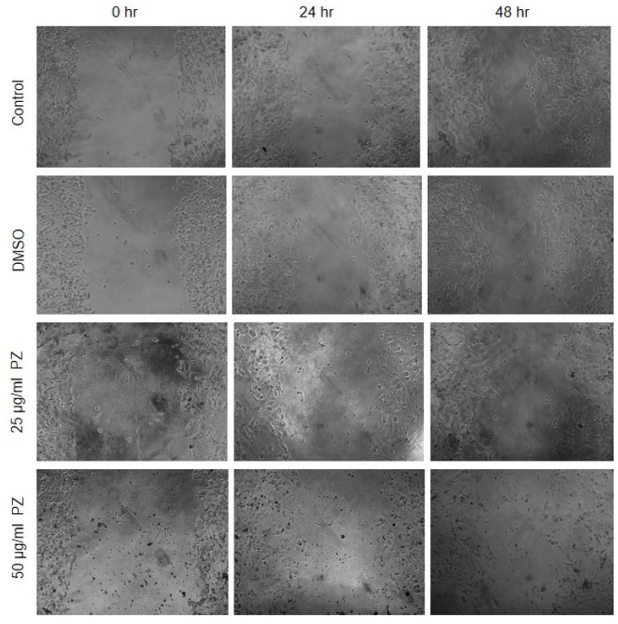

Fig. 5. *Plumbago zeylanica* leaf ethanolic extract suppresses the migration of HeLa cells in a dose- and time-dependent manner. Representative images of cells treated with 25 and 50 µg/mL of *Plumbago zeylanica* (PZ) ethanolic extracts for 24 and 48 h. Untreated control and DMSO-treated cells exhibited progressive wound closure over time, indicating normal migratory activity but PZ-treated cells showed a marked inhibition of wound closure in a dose-dependent manner (Total magnification 100X).

3.5 Methylene blue assay and Hoecst staining

The absorbance of eluted methylene blue from control and *P. zeylanica*-treated HeLa cells was comparable, indicating a similar population of adherent cells in both groups (**Fig. 7A**). However, morphological examination of the stained cells revealed concentration-dependent changes characterized by increased nuclear staining, accompanied by cell rounding and progressive cytoplasmic disintegration. At extract concentrations of 100 and 200 µg/mL, pronounced cytoplasmic granularity, membrane blebbing, and loss of membrane integrity were observed, indicating that the *P. zeylanica* extract induced significant cellular stress in HeLa cells (**Fig. 7B** and **7C**).

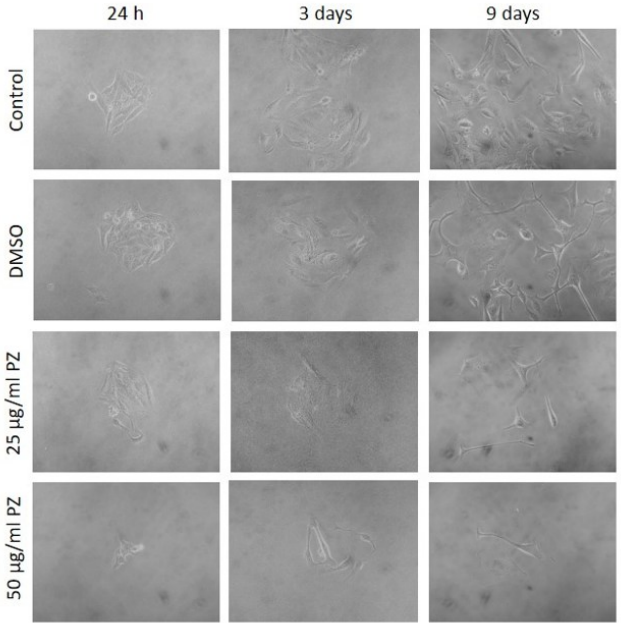

Fig. 6. *Plumbago zeylanica* leaf ethanolic extract inhibits the clonogenic potential of HeLa cells in a dose-dependent manner. Representative images of the colony formation assay showing HeLa cells treated with 25 and 50 µg/mL of *Plumbago zeylanica* (PZ) ethanolic leaf extract. Un-treated control and DMSO treated HeLa cells formed large colonies after 9 days of incubation, whereas PZ-treated cells showed a marked reduction in colony forming potential in a dose-dependent manner. Images were captured at 200× total magnification; scale bar = 150 µm.

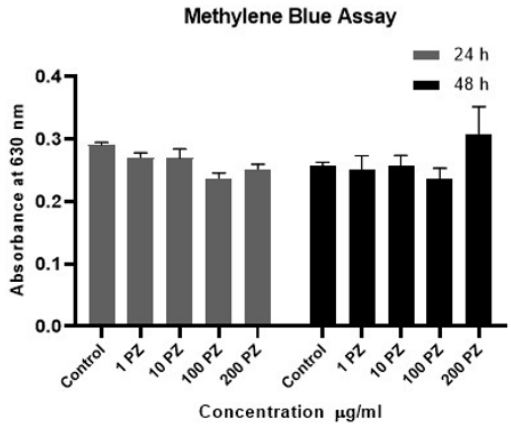

Fig. 7A. *Plumbago zeylanica* leaf ethanolic extract does not affect cell adherence as determined by methylene blue retention assay. The retention of methylene blue stain was assessed to evaluate the adherent cell population of HeLa cells following treatment with *Plumbago zeylanica* (PZ) ethanolic leaf extract. The absorbance of the eluted methylene blue dye from PZ-treated cells was

comparable to that of untreated control cells, indicating that treatment did not significantly alter the population of adherent cells under the experimental conditions. Data are presented as the mean ± SD of independent experiments.

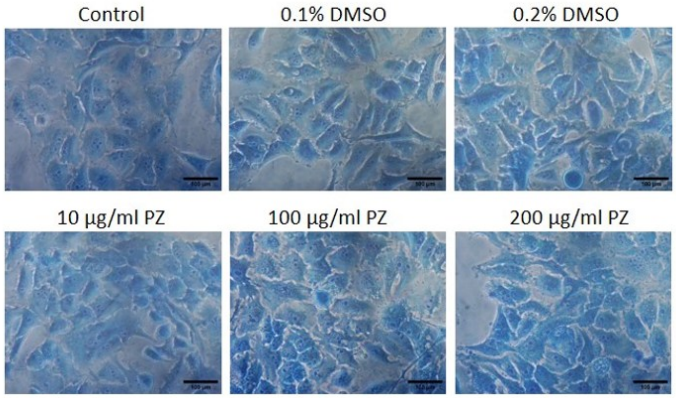

Fig 7B. Methylene blue staining reveals morphological alterations in HeLa cells following treatment with *Plumbago zeylanica* leaf ethanolic extract. Representative bright-field micrographs of methylene blue-stained HeLa cells showing the morphological changes induced by treatment with *Plumbago zeylanica* (PZ) leaf ethanolic extract. Compared with untreated control cells, PZ-treated cells exhibited enhanced nuclear staining, cell rounding, loss of normal cellular morphology, and cytoplasmic disintegration, indicative of reduced cellular integrity and cytotoxic effects. Images were acquired at 400× magnification; scale bar = 100 µm.

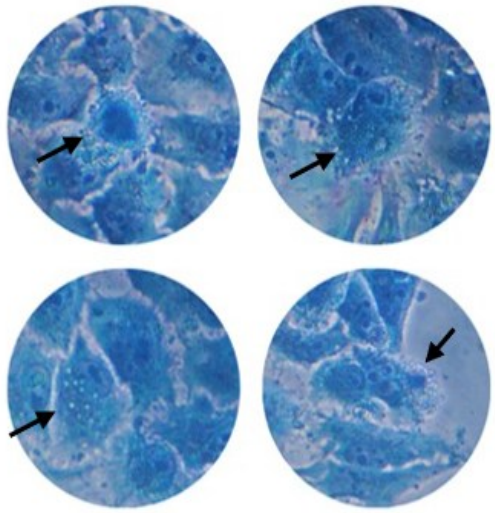

Fig. 7C. Cytological alterations in methylene blue-stained HeLa cells following treatment with *Plumbago zeylanica* leaf ethanolic extract. Representative micrographs of magnified (Zoom-In) images show the morphological changes observed in HeLa cells after treatment with 100 µg/mL and 200 µg/mL PZ ethanolic extract. Arrows indicate characteristic cytotoxic features, including increased cytoplasmic granularity, membrane blebbing, and loss of plasma membrane integrity in PZ-treated cells.

Furthermore, Hoechst staining demonstrated a modest increase in apoptotic cells following treatment with 200 µg/mL *P. zeylanica* extract. The percentage of apoptotic cells increased to 4.17% compared with the control group (**Fig. 8A** and **8B**), suggesting that the extract possesses the ability to induce apoptotic cell death in HeLa cells.

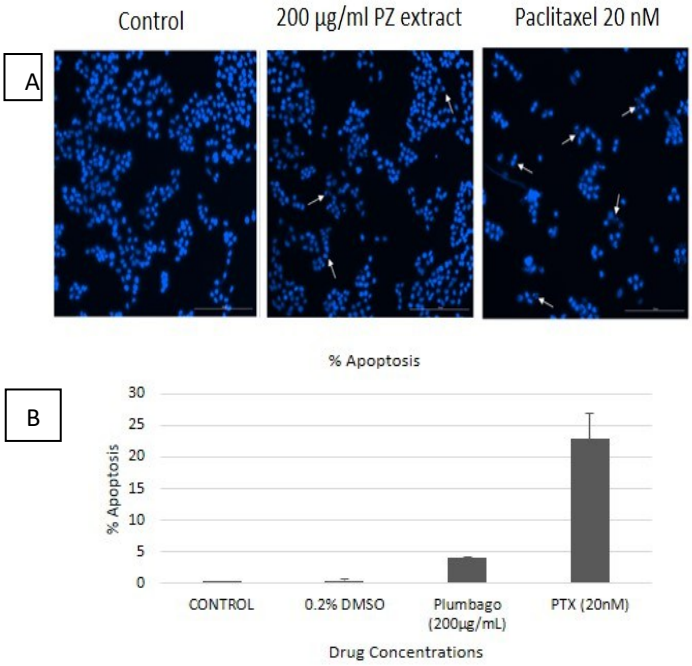

Fig. 8. *Plumbago zeylanica* leaf ethanolic extract induces apoptotic cell death in HeLa cells. (A) Representative fluorescence micrographs of Hoechst-stained HeLa cells following treatment with 200 µg/mL *P. zeylanica* (PZ) leaf ethanolic extract. Compared with untreated control cells, treated cells exhibit an increased number of apoptotic nuclei characterized by condensed and brightly fluorescent chromatin. Apoptotic cells are indicated by arrows. (B) Quantitative analysis of apoptosis showing that treatment with 200 µg/mL PZ extract resulted in an apoptotic population of 4.17%, compared with the control group. Data are presented as the mean ± SD of independent experiments.

4. Discussion

Although *P. zeylanica* is a well-recognized medicinal plant, research has predominantly focused on its roots because of their high plumbagin content and established pharmacological activities (Chaudhari et al., 2018; Mukherjee et al., 2025). In contrast, the therapeutic potential of the leaves remains comparatively underexplored, with most previous investigations limited to phytochemical profiling and antimicrobial activity (Beyene et al., 2020; D. Roselin & B. R, 2019; Latheef et al., 2024; Singh et al., 2017). The present study therefore aimed to evaluate the antioxidant and anticancer potential of the ethanolic leaf extract of *P. zeylanica*.

The phytochemical profile suggests that the antioxidant capacity of the leaf extract is primarily associated with flavonoids and other reducing phytoconstituents rather than phenolic compounds. Although the total phenolic content was comparatively low, the extract exhibited appreciable ferric reducing antioxidant power and reducing capacity, indicating the presence of compounds capable of donating electrons to neutralize reactive species. Similar observations of relatively low phenolic content in *P. zeylanica* leaves compared with roots have been reported by Sundari et al., (2017), whereas Beyene et al., (2020) reported substantially higher phenolic and flavonoid contents in methanolic leaf extracts. These differences are likely attributable to variations in extraction solvent, geographical origin, plant maturity, and extraction methodology, all of which are known to influence phytochemical

recovery.

The antioxidant assays demonstrated that the extract exhibited significantly higher ABTS radical scavenging activity (75.1%), relatively moderate DPPH activity (26.8%) and relatively low superoxide radical scavenging activity (18.88%). The stronger ABTS activity suggests that the *P. zeylanica* ethanolic extract is rich in hydrophilic antioxidants with strong electron-donating capacity, while moderate DPPH activity is limited to lipophilic compounds (Ácsová et al., 2019; Arnao et al., 2001). Lower superoxide scavenging activity implies limited efficacy against biologically relevant ROS. In another study by Polaki et al., (2023), the methanolic extract of P. zeylanica leaves exhibited DPPH radical scavenging activity of 80% and FRAP activity of 100 mg GAE/g, thereby suggesting that the differences in extraction solvent, extraction method, phytochemical composition, and environmental factors affects secondary metabolite accumulation.

Although the biological activities of Plumbago roots have been extensively investigated, the anticancer potential of Plumbago leaves remains largely unexplored. Previous reports have demonstrated anticancer effects of *P. zeylanica* leaves against Ehrlich Ascites Carcinoma in experimental animal models (Sachin Hiradeve et al., 2010) and anti-proliferative activity of plumbagin-rich leaf extracts from P. indica and P. zeylanica (field-grown and in vitro cultures) against AGS (stomach cancer) and MDA-MB-231 (breast cancer) cell lines (Jayanthi et al., 2020). In another study, (Sundari et al., 2017) reported highest cytotoxic potency (IC_{50} = 164.5 µg/mL) of Plumbago root extract compared to leaf extract (IC_{50} = 274.9 µg/mL) and stem extract (IC_{50} = 379.5 µg/mL) against A549 human lung carcinoma cells. Collectively, these findings suggest that although the roots possess greater cytotoxic activity, Plumbago leaves also exhibit promising anticancer potential that warrants further investigation.

In the present study, the *P. zeylanica* ethanolic leaf extract exerted a greater influence on the proliferative and migratory behavior of HeLa cells than on cell viability. While only a modest reduction in metabolic activity and apoptosis was observed at the tested concentration, the extract exhibited dose-dependent reduction in cell proliferation, migration, and wound closure compared to the control. At 50 µg/mL concentration, the extract significantly reduced cell migration and markedly suppressed wound closure. This inhibitory effect on cell migration may be attributed to plumbagin which is reported to influence actin cytoskeletal dynamics and suppress signaling pathways associated with metastasis (Kang et al., 2017; Wang et al., 2023). The clonogenic assay also demonstrated a dose-dependent decline in the colony-forming capacity of HeLa cells, as evident by reduced colony size and scattered colony formation. These observations suggest that the extract primarily restricts the long-term proliferative and migratory capacity of HeLa cells rather than inducing extensive acute cytotoxicity. Such effects are therapeutically relevant because inhibition of migration and clonogenic growth is associated with reduced metastatic potential and decreased tumour recurrence.

Although cell cytotoxicity studies demonstrated a marginal reduction in cell viability, a notable morphological alterations were observed in cells treated with 200 µg/mL extract, wherein cells exhibited a striated and rounded appearance, suggesting that the *P. zeylanica* extract induced cellular stress responses in HeLa cells. Further staining with methylene blue revealed additional morphological alterations including cell rounding, increased cytoplasmic granularity, membrane blebbing, and cytoplasmic disintegration in HeLa cells further supporting the induction of cellular stress. Despite these cytological changes, methylene blue staining indicated a substantial proportion of cells that remained adherent and Hoechst staining demonstrated only modest apoptosis in HeLa cells. These findings do not exclude the possibility that the extract induces alternative mechanisms of growth inhibition, such as cell cycle arrest, senescence, or non-apoptotic stress responses. Similar concentration-dependent cytotoxic effects have been reported by Singh et al., (2017), although substantially higher extract concentrations were employed in HCT-116 and K-562 cells, emphasizing that differences in cell type, extract composition, and treatment conditions influence the observed biological response.

The Plumbagin, a naphthoquinone derivative of *P. zeylanica*, is a major bioactive compound of pharmacological properties such as anti-diabetic and anti-cancer (Chen et al., 2016). It exhibits anticancer activity through pro-oxidant mechanisms by increasing reactive oxygen species (ROS), leading to mitochondrial dysfunction, DNA damage, oxidative stress, lipid peroxidation and activation of apoptotic pathways (Powolny & Singh, 2008; Srinivas et al., 2004). It also disrupts intracellular antioxidant defenses, including glutathione (GSH), thioredoxin reductase (TrxR), and glutathione reductase (GR), thereby amplifying oxidative stress (Hwang et al., 2015). However, because plumbagin is present in much lower concentrations in leaves (0.00007%), than in roots (1.3%) (Sundari et al., 2017), the biological effects observed in the present study are likely mediated not only by plumbagin but also by flavonoids and other phytoconstituents acting individually or synergistically. This may explain why the leaf extract exhibited pronounced anti-proliferative and anti-migratory effects against HeLa cells despite relatively modest cytotoxicity. Overall, the findings demonstrate that the ethanolic leaf extract of *P. zeylanica* possesses moderate anticancer potential characterized primarily by anti-proliferative and anti-migratory activities, supporting further investigation of its phytochemicals as potential chemopreventive or chemosensitizing agents.

5. Conclusion

The ethanolic extract of *P. zeylanica* leaves showed high levels of total flavonoids, and also demonstrated pronounced FRAP activity, Reducing power, and significant ABTS radical-scavenging capacity. The *P. zeylanica* leaf extract exhibited moderate anticancer activity against HeLa cells, characterized by significant inhibition of cell migration and clonogenic growth, accompanied by morphological alterations indicative of cellular stress and a modest reduction in cell viability and induction of apoptosis. These findings suggest that the extract primarily exerts anti-proliferative and anti-migratory effects under the experimental conditions tested. These features potentiates the role of *P. zeylanica* leaves extract as chemosensitizing agent and promising candidate as chemopreventive agent requiring further investigation. Further work needs to be done to confirm the *P. zeylanica* -induced cellular stress, assess the cell cycle arrest phases and identify the bioactives in addition to plumbagin and confirm their role as prooxidants capable of inducing apoptotic cell death in cancer cells. Further, extensive work on varied tumor cell lines, selective toxicity studies and *in vivo* experiments could help to bring out the application of *P. zeylanica* leaf extracts for anticancer therapies.

Conflict of interest

No declared

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Authors contribution

Study designing, conceptualization and project supervision, MH and SK; Standardization and validation of antioxidant assay, SK, YG and JJ; Formulating cell lines based studies, MH, YG and JJ; Original draft

preparation, MH and YG, Review and editing drafts of manuscript, statistical analysis of all data, MH; Funding acquisition, YG and JJ. All authors read and approved the final manuscript.

Ethical statement

Not Applicable

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

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