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Evaluation of Antioxidant Potential and Selective Cytotoxicity of Endangered Medicinal Plant *Prunus ceylanica* (Wight) Miq.

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ABSTRACT

Cancer is still a major public health challenge, accounting for one out of every six deaths. Compounds with antioxidant capabilities are especially interesting because they may have anticancer qualities by neutralizing free radicals that can cause DNA damage and contribute to cancer. The antioxidant capacities (DPPH and FRAP assays) and cytotoxic activities (MTT assay) of human adenocarcinoma cells (PC-3) and mouse fibroblast cells (L-929) of *Prunus ceylanica* were studied. Results revealed that the highest antioxidant activities were shown in the methanol bark extract (IC50 = 40.19±0.75 µg/ml), while using the DPPH method and the acetone bark extract (IC50 = 15.77±1.25 µg/ml) through the FRAP assay. Furthermore, the acetone bark extract exhibited the highest total phenolic content (447.61±7.17 mg GAE/g dry extract). Remarkably, the acetone bark extract demonstrated significant specificity against PC-3 cells ($P < 0.005$) with the lowest IC50 value (27.74±1.96 µg/ml) and minimal toxicity to L-929 cells (IC50=175.08±0.01 µg/ml). These results suggested a possible approach for the future development of a novel anticancer medication against lethal adenocarcinoma.

Key words: Adenocarcinoma cell line, DPPH assay, fibroblast cell line, FRAP assay, MTT assay

INTRODUCTION

Plants are a great source of several bioactive substances, including phenolic compounds, which have drawn a lot of attention because of their potential health advantages. Research into new pharmaceuticals to promote healthy living for both humans and animals is guided by traditional folk remedies from plants (Syam *et al.*, 2019, Sandhya *et al.*, 2021). Numerous analytical techniques are used to determine the total phenolic content and antioxidant activity of plants, including the DPPH (2, 2-diphenyl-1-picrylhydrazyl) and FRAP (ferric ion reducing antioxidant power) assays for measuring the antioxidant activity and the Folin-Ciocalteu method for measuring total phenolic content.

Antioxidants are compounds used to protect the cells from oxidative damage caused by reactive oxygen species (ROS) such as free radicals, peroxides, singlet oxygen, hydroxyl radicals and nitric oxide radicals. Antioxidants neutralize these harmful free radicals, thereby reducing the risk of oxidative damage to cells and tissues (Lourenco *et al.*, 2019). Researchers and pharmaceutical industries have intensified their efforts to identify and utilize plants rich in phenolic compounds for the development of natural antioxidant-based supplements, functional foods and pharmaceutical formulations. Exploring the significance of these plant-derived antioxidants in improving human health and disease prevention has gained popularity.

Cancer is the most lethal disease, coming in third place after cardiovascular and infectious diseases. More than 100 different types of cancer have been reported as a result of defects in the molecular pathways. An epithelial benign cancer, Adenocarcinoma, is a frequent human cancer that occurs mostly in the urogenital tract, lungs, liver and gastrointestinal tracts. Prostatic adenocarcinoma is the most common type of cancer that affects the epithelial cells of the prostate gland and accounts for 6.8% of cancer deaths in men. Prostate cancer is a significant global health concern, particularly in aging populations, and its prevalence is expected to rise in the coming years (Sung *et al.*, 2021). Research continues to expand our understanding of the biology and genetics of prostatic adenocarcinomas and the need to find

novel natural anticancer agents to avoid the adverse effect of artificial drugs. Plants are the potential source of natural compounds due to their safe and inexpensiveness. Around the world, nearly 75% of people depend on plants either directly or indirectly for their medical requirements.

Prunus ceylanica, commonly known as Ceylon almond, is a species belonging to the Rosaceae family. This evergreen tree is native to the highland forests of Sri Lanka and Eastern, Deccan and Peninsular India. However, due to habitat destruction and other anthropogenic activities, its natural populations have been significantly reduced, leading to its classification as an endangered species. Various phytochemical studies have identified the presence of bioactive compounds such as flavonoids, phenolic acids, tannins and alkaloids. As *Prunus ceylanica* faces the threat of extinction, conservation efforts are essential to protect and preserve this valuable plant species. In addition to the conservation aspect, further research on its taxonomy and phytochemistry can contribute to our understanding of its ecological importance and potential benefits to human health. The current study mainly aimed at evaluating the total phenolic content, antioxidant potentialities and anticancer potential of acetone, methanol and ethylacetate extracts of *Prunus ceylanica* (Wight) Miq bark and leaf on human prostate adenocarcinoma cell line PC-3 and general toxicity on mouse fibroblast cell line L-929 through MTT assay.

MATERIALS AND METHODS

In August 2022, the plant was gathered from the Maredumilli forest region in Andhra Pradesh, India (17°38′35.6″ N 81°45′45.0″ E). The botanical identification of the plant material was performed by the Department of Botany, Andhra University, Visakhapatnam. A voucher specimen (number 25528 AUV) was deposited in the Andhra University Herbarium for future reference.

Fresh plant materials (leaves and bark) were collected and subjected to shade-drying to obtain a dried sample. The dry material was finely pulverized to a mesh size of 60 mm using an electronic blender before being utilized for solvent extraction. For sample preparation, 100 g of dried material was extracted with 500 ml of each solvent (methanol, acetone, and ethyl acetate) at 45°C for 24 h by using Soxhlet apparatus.

Folin-Ciocalteu reagent was used to assess the sample's total phenolic content. A calibration curve ranging from 25 to 500 µg/ml was prepared using gallic acid as the reference standard. To perform the test, 0.1 ml of the sample was combined with 2 ml of Folin-Ciocalteu reagent before being neutralized with 4 ml of 7.5% sodium carbonate solution (w/v). The absorbance of the subsequent blue colour was measured at 765 nm using a double-beam UV/VIS Spectrophotometer LMSP-UV-1200-PC. The total phenolic content was determined using the linear equation derived from the gallic acid standard curve. The total phenolic compounds in the sample were reported as mg/l gallic acid equivalent (GAE).

The antioxidant capacity of the fractions was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. The stock solution was made by dissolving 24 mg of DPPH in 100 ml of methanol and storing it at -20°C until needed. Following that, a 3 ml portion of the working DPPH solution was combined with 100 µl of the sample at various concentrations (20, 40, 60, 80 and 100 µg/ml). The mixture was properly mixed and allowed to settle at room temperature for 30 min. The absorbance at 517 nm was then measured using a UV-VIS Shimadzu spectrophotometer. The same process was used to make a control, but no sample was added. The standard was ascorbic acid, which was used at the same concentrations. The following formula was used to determine the percentage of scavenging activity :

$$\text{Per cent inhibition} = \frac{(\text{Control absorbance} - \text{Sample absorbance})}{\text{Control absorbance}} \times 100$$

Acetate buffer (pH 3.6), 20 mM TPTZ solution (including 40 mMHCl) and 20 mM $FeCl_3$ were combined in a 10:1:1 ratio to create a FRAP solution. The extracts were then added to the FRAP solution at a ratio of 1:30, and the mixture was left to react at 37°C in the dark for 30 min. The resulting blue-coloured product, known as the Ferroustripyridyltriazine complex, was measured for absorbance spectro-

photometrically at 593 nm. Ascorbic acid was used as a standard at various concentrations (ranging from 20-100 µg/ml).

The culture medium DMEM was supplemented with 10% foetal bovine serum from Himedia, and 1X Antibiotic Antimycotic solution. At 37°C and 5% CO_2, the cells were kept in a CO_2 incubator. The cells were then treated for 24 h with various concentrations of test samples (AL, AB, ML, MB, EB and EL) and positive control (30% DMSO) in a serum-free medium. After the treatment, the medium was removed, and 0.5 mg/ml MTT in 1X PBS was added to each well. The cells were then maintained in a CO_2 incubator at 37°C for 4 h. The MTT-containing solution was discarded, and the cells were washed in 200 µl of PBS. To dissolve the crystals that had developed, 100 µl of DMSO was added and thoroughly mixed. The formazan dye's colour intensity was measured at 570 nm, where it showed a purple-blue hue. A microplate reader was used to measure absorbance at 570 nm.

RESULTS AND DISCUSSION

This investigation was aimed at assessing the total phenolic content (TPC), antioxidant activities through DPPH and FRAP assays, and the cytotoxic potential on L-929 and PC-3 cell lines using MTT assay.

Fig. 1 shows the total phenolic content (TPC), which was calculated using the linear curve obtained from the standard gallic acid (Fig. 2; Y = 0.0058x + 0.3205; R^2 = 0.9886), in the plant extracts of ethyl acetate, acetone and methanol. The TPC was expressed in mg GAE/ g of dry extract. Regarding the bark extracts, the acetone extract exhibited the highest TPC (447.61±7.17), followed by the methanol extract (443.36±0.0), with the lowest TPC being reported in the ethyl acetate extract (413.79±0.0). For the leaf extracts, the acetone extract also showed the highest TPC (430.23±4.95), followed by the methanol extract (336.26±2.31), and the lowest TPC was found in the ethyl acetate extract (308.7±8.26). The bark acetone extract had the highest TPC (447.61±7.17), while the lowest was observed in the ethyl acetate leaf extract (308.7±8.26). Furthermore, when comparing leaf and bark extracts, it was evident that bark extracts contained higher amounts of TPC. Additionally, the acetone solvent demonstrated

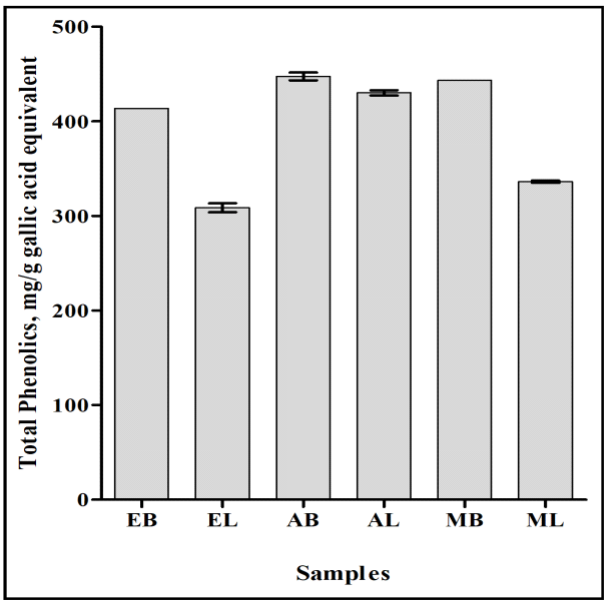

Fig. 1. Total phenolic content of various extracts of *Prunus ceylanica*.

EB: Bark ethyl acetate extract, EL: Leaf ethyl acetate extract, AB: Bark acetone extract, AL: Acetone leaf extract, MB: Bark methanol extract and ML: Leaf methanol extract.

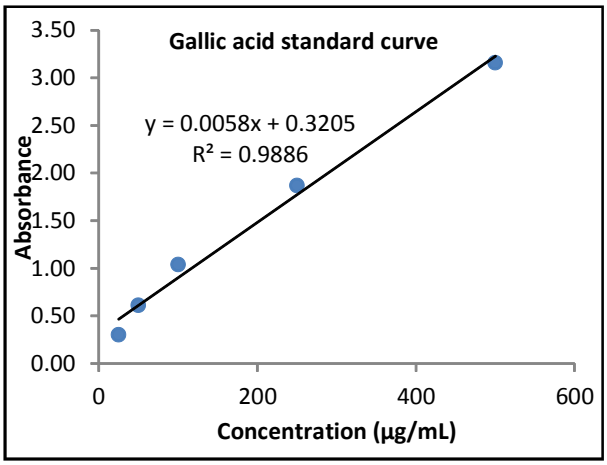

Fig. 2. Gallic acid standard graph for the determination of total phenolic content.

high efficiency in solubilizing phenolic compounds from the plant materials.

Antioxidants are chemicals that protect live cells from possible harm like cancer by stabilizing free radicals produced during the oxidation process. In this investigation, ethyl acetate, acetone and methanol were used as solvents to assess the antioxidant activity of bark and leaf extracts from *P. ceylanica*. To assess radical scavenging activity, the DPPH standard radical was employed. The results revealed that the different solvent extracts had a significant impact on the DPPH radical. The

crude methanol extract from the bark exhibited the highest radical scavenging activity across all tested concentrations, ranging from 31.41±1.46 to 83.17±4.25. Conversely, the lowest percentage of inhibition was observed in the acetone leaf extract, ranging from 21.90±0.57 to 77.12±1.25 (Figs. 3 and 4).

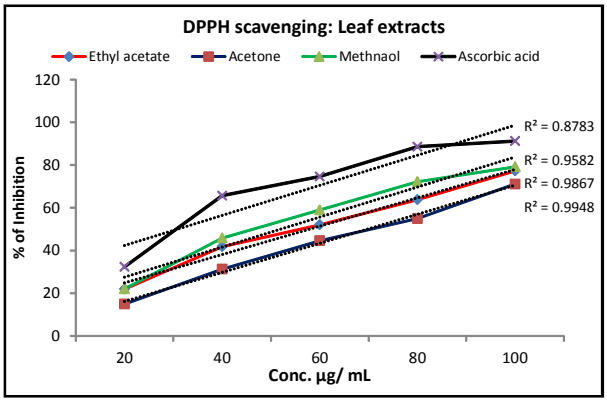

Fig. 3. Percentage of RSA in DPPH activity of leaf extracts of *P. ceylanica*.

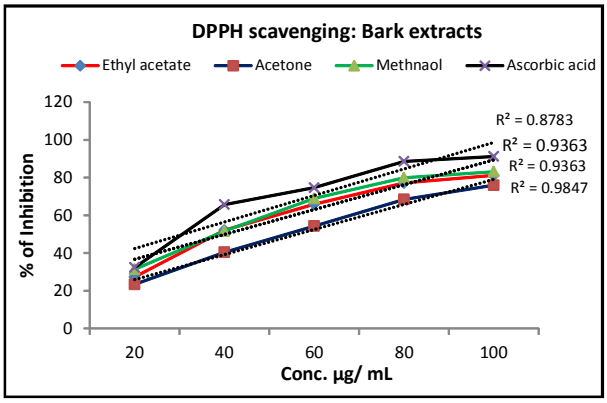

Fig. 4. Percentage of RSA in DPPH activity of bark extracts of *P. ceylanica*.

At all concentrations tested, the methanol extract of the leaf showed the highest percentage of inhibition (ranging from 22.02±0.57 to 79.19±2.12), followed by the ethyl acetate extract (ranging from 21.9±0.75 to 79.29±1.57), and the acetone extract (ranging from 14.93±0.57 to 71.12±1.5). For the bark extracts, acetone extract had the lowest percentage of inhibition (ranging from 23.4±0.57 to 73.97±1.5), followed by ethyl acetate (ranging from 27.32±0.75 to 81.21±1.57) and methanol (ranging from 31.41±1.46 to 83.17±4.25). In general, methanol extracts exhibited higher antioxidant activities compared to ethyl acetate and acetone extracts, although none of them surpassed the standard positive control (ascorbic acid; $P < 0.05$).

As shown in Figs. 3 and 4, the DPPH radical scavenging activity of the extracts increased as their concentration rose from 20, 40, 60, 80 and 100 µg/ml. The IC50 value, representing the substrate concentration at which 50% of the DPPH activity is lost, determines the antioxidant activity, with lower IC50 values indicating greater antioxidant potential. The methanol extract of the bark exhibited the lowest IC50 value (40.19±0.75 µg/ml), while the ethyl acetate extract of the leaf showed the highest IC50 value (57.94±1.625 µg/ml). Across all the solvents used, the bark extracts demonstrated higher antioxidant activity compared to the leaf extracts. Notably, all the extracts displayed significant ($P < 0.05$) and promising antioxidant capabilities. As a standard, ascorbic acid showed greater antioxidant activity as evident by its IC50 value of 24.73±0.57 µg/ml, as presented in Table 1.

The methanolic extract of *P. ceylanica* leaves demonstrated remarkable antioxidant activity in the FRAP radical scavenging assay. Among all the leaf extracts, the crude methanol extract exhibited the highest percentage of inhibition at all tested concentrations, ranging

Table 1. IC 50 values of antioxidant and anti-cancer activities

Plant extracts	IC50 values for antioxidant activities		IC50 values for cytotoxicity analysis	
	DPPH assay	FRAP assay	L-929	PC-3
EB	43.84±0.75	19.98±0.57	296.16±2.2	31.6±1.77
EL	57.94±1.62	25.33±0.75	536.16±1.91	64.04±1.38
AB	41.25±1.53	15.77±1.25	175.08±0.01	27.74±1.96
AL	49.75±2.12	20.33±1.25	355.05±0.05	45.35±1.47
MB	40.19±0.75	16.05±0.75	232.68±0.02	29.6±1.7
ML	51.98±1.25	20.76±1.23	457.68±0.09	42.97±1.79

Where, EB: Bark ethyl acetate extract, EL: Leaf ethyl acetate extract, AB: Bark acetone extract, AL: Acetone leaf extract, MB: Bark methanol extract and ML: Leaf methanol extract. All values given as mean±Standard deviation for (n=3). Units: µg/ml.

from 48.61±1.64 to 88.83±3.57, followed by the ethyl acetate extract (49.43±1.75 to 84.65±4.25). Conversely, the lowest percentage of inhibition was reported in the acetone extract (28.42±0.57 to 79.41±1.25). In the case of bark extracts, the methanol extract also exhibited the highest percentage of inhibition (ranging from 54.99±1.27 to 90.11±4.12), followed by the ethyl acetate extract (ranging from 52.99±0.75 to 88.93±1.57), and the acetone extract showing the least inhibition (ranging from 24.15±2.57 to 81.87±3.5) at all tested concentrations. Moreover, as the plant extract concentration increased from 20 to 100 µg/ml (Figs. 5 and 6), the FRAP radical scavenging activity also increased. In terms of IC50 values, the acetone extract of bark displayed the lowest value (15.77±1.25 µg/ml), followed by the methanol extract of bark (16.05±0.75 µg/ml) while the highest IC50 value was reported in the ethyl acetate leaf extract (25.33±0.75 µg/ml). All extracts demonstrated significant ($P < 0.05$) and noteworthy antioxidant capabilities. As for the standard, ascorbic acid exhibited an IC50 value of 6.67±0.57 µg/ml (Table 1).

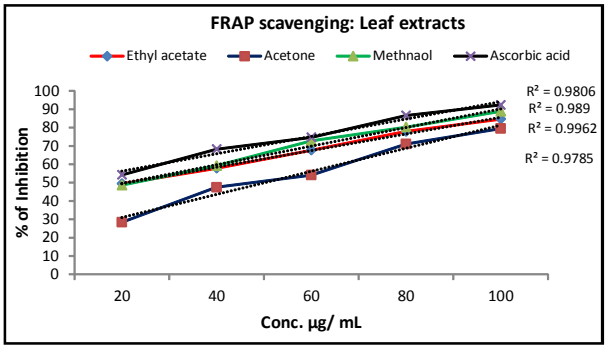

Fig. 5. Percentage of RSA in FRAP activity of leaf extracts of *P. ceylanica*.

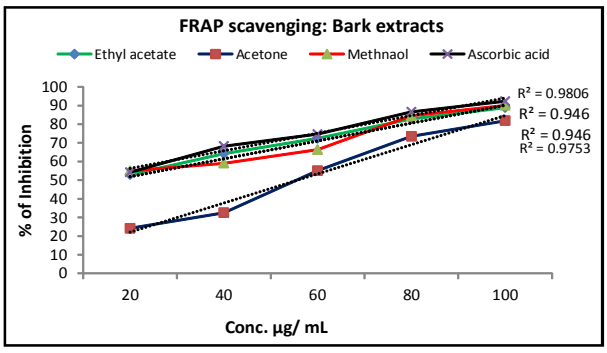

Fig. 6. Percentage of RSA in FRAP activity of bark extracts of *P. ceylanica*.

Leaf and bark extracts of *P. ceylanica* were examined for their total phenolic content, antioxidant potential and cytotoxic effects on the PC-3 and L-929 cell lines. Due to their higher metabolic rate, cancer cells heavily rely on elevated levels of reactive oxygen species (ROS) for proliferation and metastasis (Qian *et al.*, 2019). Compounds possessing antioxidant properties may demonstrate anticancer abilities by neutralizing ROS. This research helps in understanding the possible therapeutic implications of *P. ceylanica* extracts in controlling cancer and oxidative stress-related disorders.

In the present investigation acetone, methanol and ethyl acetate extracts of the bark and leaf of *P. ceylanica* showed elevated levels of TPC and antioxidant activities. When compared to leaf bark extracts showed higher antioxidant potentialities due to the presence of polyhydroxy or polyphenolic compounds as an impact on the scavenging ability of plant extracts. Two cultivars of domesticated plum (*P. domestica*); African Rose and Santa Rosa, had ethanolic extracts that had high antioxidant effects, with IC50 values of 13.923 and 18.416 g/ml, respectively (El-Beltagi *et al.*, 2018). According to Dashtizadeh *et al.* (2021), green-synthesized CuNPs had low antioxidant activity (15.90% inhibition) in the DPPH test but it had high potential cytotoxic activity (LC50 = 3.6 µg/ml) in brine shrimp lethality assay. From this literature, one can conclude that the genus *Prunus* has a good amount of phenolic compounds and antioxidant molecules, further analysis is required to identify novel antioxidant compounds.

The cytotoxicity of the chosen plant samples against the human prostatic adenocarcinoma cell line (PC-3) and mouse fibroblast cell line (L-929) was assessed using the MTT assay, and the findings are summarized in Table 1. The results revealed a concentration-dependent cytotoxicity, and among all the tested plant extracts, the bark extracts of acetone exhibited the highest anticancer activity against the PC-3 cell line (IC50 = 27.74±1.96 µg/ml), while the lowest activity was reported in leaf extracts of ethyl acetate (IC50 = 64.04±1.38 µg/ml). The bark extracts demonstrated significant anticancer activity compared to the leaf extracts. Additionally, for all the tested plant extracts, the leaf ethyl acetate extract displayed the lowest cytotoxic activity against the L-929 cell line (IC50 = 536.16±1.92 µg/ml), while the highest cytotoxic activity was reported in the acetone bark extract (IC50=175.08±0.01 µg/ml).

The test results for cytotoxic activities revealed that the plant extracts were significantly (P 0.05) cytotoxic to PC-3 cells in a dose-dependent manner, whereas normal fibroblast cells (L-929) were far less sensitive. The substantially higher IC-50 value of leaf ethyl acetate extract (536.16±1.92 g/ml) on L-929 cells confirmed the extract's relative nontoxicity and safety to normal cells. The morphological study demonstrated that the extract had a significant effect on the morphology of PC-3 cells when compared to negative control-treated cells. The cells shrunk abnormally, in contrast to normal L-929 cells, which revealed only minor changes with their vehicle-treated counterparts (Figs. 7 and 8). These data imply that the extract's toxicity was very selective to adenocarcinoma cells, while normal fibroblast cells were non-toxic at a certain dose. A significant positive correlation was observed

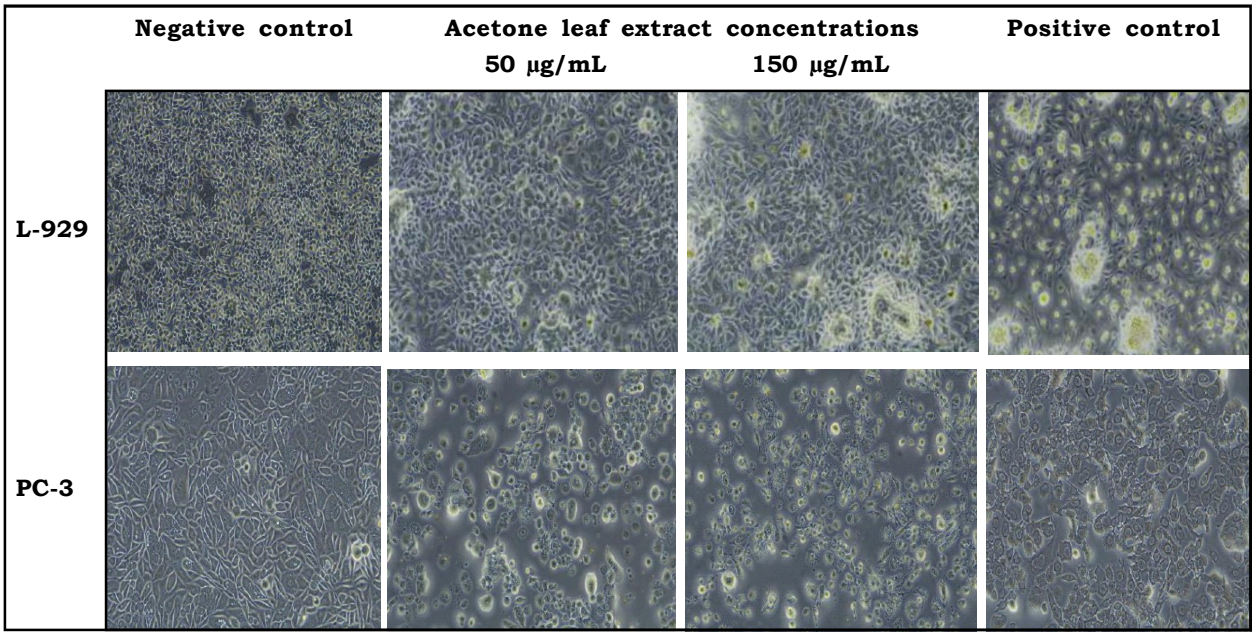

Fig. 7. The phase contrast image indicates considerable morphological alterations in PC-3 cells treated with *P. ceylanica* acetone leaf extract compared to normal cell L-929.

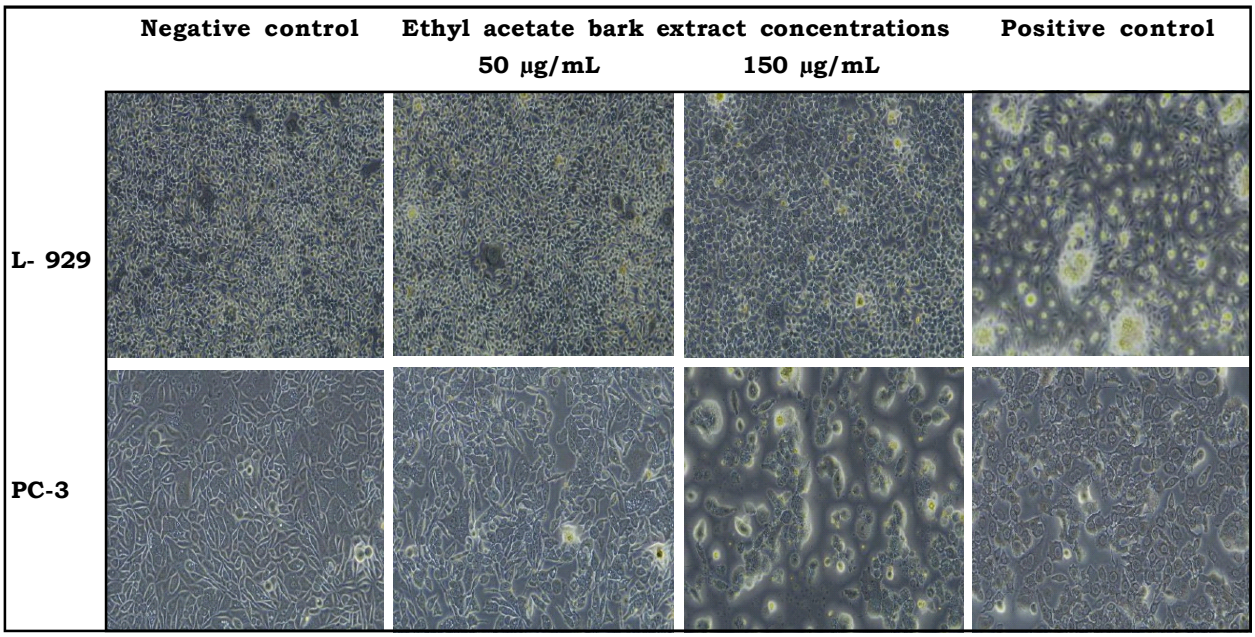

Fig. 8. The phase contrast image indicates considerable morphological alterations in PC-3 cells as compared to normal cell L-929 after treatment with *P. ceylanica* ethyl acetate bark extract.

Table 2. Correlations between *P. ceylanica* phenolic content and IC50 values in antioxidant and cytotoxicity experiments

Assays	Correlation (R^2)	R-value	Standard error	Significance (P value)
DPPH assay	0.803*	0.737	33.61	0.039
FRAP assay	0.775*	0.669	35.94	0.048
Cytotoxicity against L-929 cell line	0.880*	0.840	60.11	0.018
Cytotoxicity against PC-3 cell line	0.691	0.588	42.10	0.080

*Significant P value <0.05.

between TPC and IC50 values for cytotoxicity screening against fibroblast cell line L-929 (MTT assay), DPPH assay and FRAP reducing power activities (R^2=0.880, R^2=0.803 and R^2=0.775, respectively), while no significant correlation was observed between TFC and IC50 values of anticancer activity screening against PC-3 cell line (Table 2). P values less than 0.05 were considered significant for this correlation assay.

P. ceylanica had powerful cytotoxic action against the cancer cell line PC-3 and only mild toxicity towards the normal fibroblast cell line L-929. According to Celik *et al.* (2023), the *P. divaricata* fruit's methanol extract had a cytotoxic effect at dosages of 20 mg/ml on the lung cancer cell lines A549 and H1299 but not on healthy lung cells Beas-2b. By lowering the expression of the genes KRAS and PIK3CA, this extract induced apoptosis in cancer cells. *P. domestica* had been shown to have anti-cancer activity against human lung cancer (A549), colorectal adenocarcinoma (Caco-2), and human breast cancer (MCF-7) (El-Beltagi *et al.*, 2018). *P. spinosa* had also been shown to have an anticancer effect against the colorectal cancer cell line HCT116 (Condello *et al.,* 2019). The anticancer potential of *P. armeniaca* against various tumors was summarized by Kitic *et al.* (2022). Previous studies established the anticancer activity of the genus *Prunus*, although *P. ceylanica* was not tested for cytotoxicity. The findings given here offered reliable evidence that *P. ceylanica* contained interesting anti-adenocarcinoma compounds that should be investigated further in the future for understanding and potential therapeutic development.

CONCLUSION

P. ceylanica's bark and leaf extracts both contain high concentrations of phenolic compounds, which support the plant's antioxidant and anticancer potential. Particularly, the acetone and methanol extracts from the bark showed promising anticancer outcomes, while the methanol and ethyl acetate extracts from the bark had high antioxidant properties. Moreover, all extracts displayed concentration-dependent cytotoxicity, with the bark extracts of acetone and methanol showing significant cell lysis. Based on *in vitro* experiments, *P. ceylanica* demonstrated antioxidant and anticancer activities against the adenocarcinoma cell line. However, further research is essential to test these results *in vivo* and to gain a deeper understanding of the molecular mechanisms of action involved.

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