Pharmacogn. Res., 2024; 16(4):718-726.
https://www.phcogres.com

Original Article

Screening of *in vitro* Anticancer Activity of Various Human Cancer Cell Lines and Induced Apoptosis and Cell Arrest by Ethanolic Bulb Extract of *Pancratium zeylanicum*

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ABSTRACT

Aim: World widely the human society suffers from various types of cancer due to many causes. Thus, finding out a good anticancer agent is a need of the hour. Medicinal herbs are always considered at pole position whenever we discuss anticancer properties. Numerous herbal plants have been assessed and are subjected to phytochemical analysis to learn more about their anticancer effects. **Materials and Methods:** To assess the anti-cancerous activity of PZBE extract in MTT assay, cell cycle analysis, and apoptosis studies have been performed using different cancer cell lines. **Results:** This study aims to assess the impact of ethanolic extract of *Pancratium zeylanicum* Bulbs (PZBE) against cancerous cells *in vitro* conditions. MTT assay was used to compare the PZBE extract with Annexin V as a positive control compound against four human cancer cell lines, including Human cervical cancer (HeLa), colon cancer (HT-29), lung cancer (A549) and breast cancer (MCF-7) cell lines with the IC_{50} values of 209.28 µg/mL for HeLa, 133.09 µg/mL for A-549, 264.46 µg/mL for MCF-7 and 264.75 µg/mL for HT-29 cell lines. Increasing of the populations observed in G2-M and G0-G1 cells by 42% and 44.78%, respectively when compared to the control. The graph demonstrates that PZBE extracts significantly induced G0-G1 arrest in A-549 cells. Furthermore, the PZBE extract effectively induced apoptosis when compared to untreated cells, A-549 cells treated with IC_{50} concentration, and annexin-V double-staining assay demonstrated an increase in the percentage of early and late apoptotic cells. **Conclusion:** The ethanol extract of *Pancratium zeylanicum* bulbs demonstrated potent cytotoxic activity against the cancer cell lines HeLa, A-549, MCF-7, and HT-29. The current study's findings may be helpful in the development of plant-based anticancer drugs.

Keywords: Anticancer activity, HeLa, A-549, MCF-7, HT-29, Apoptosis, Ethanol extract, Annexin-V.

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Received: 04-11-2023;
Revised: 08-02-2024;
Accepted: 14-06-2024.

INTRODUCTION

Amaryllidaceae family is one of the top 20 most revered plant families for its medicinal properties with more than 1600 species spread across the globe.[1] They are bulbous flowering plants that are also used as decorative items. South Africa is home to about one-third of the species in the Amaryllidaceae family, which are commonly employed in traditional medicine which are widely used in traditional drug.[2,3] Among the traditional uses of the Amaryllidaceae are treating everything from brief illnesses like boils, headaches and coughs to more complex conditions (e.g., cancer, tuberculosis, diabetes). The antimicrobial, anti-tumoral, Antiacetyl Cholinesterase (AChE), and cytotoxic properties of Amaryllidaceae are well known.[4-12] There have been few studies carried out on West African Amaryllidaceae species such as Pancratium sp., collected in Senegal for traditional medicine.[13,14] About 20 species comprise the genus *Pancratium*, stretching across West Africa through Namibia, including throughout the Canary Islands to tropical Asia.[15]

Pancratium zeylanicum (Rain Flower) has a spherical bulb with a diameter of up to 5 cm. A rosette of 6-12 leaves emerges from the ground; they are lance- or strap-shaped, the length is up to 30 cm and the width is about 2 cm hairless and dark green. Flowers bloom singly on stems between 10 and 20 cm long. Tepals are a straight line, approximately 6.5-8 cm in length and 4-7 mm in width, tapering, narrowly lance-shaped and the flower tube is 2.5-3.5 cm long. The flowers are pure white. Stamen filaments are 3 cm long and upright. A capsule makes up the fruit. Fractures and

DOI: 10.5530/pres.16.4.83

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bruises are treated with it in folk medicine in SE Asia (Cambodia, Laos and Vietnam).

According to the World Health Organization (WHO), many nations suffer from; including developing nations, continue to use plants and products derived from natural sources for medical purposes.[16] Around the world, 60% of anticancer drugs come from natural sources.[17] The substances that come from nature are easily accessible, generally more tolerable, and thought to be safe for normal human cells.[18]

For some types of cancer, anticancer medications are viewed as the first line of treatment.[19] There are numerous anticancer medications with diverse sources and forms of action for treating different types of cancer; however, most of these medications have detrimental adverse consequences. Alkylating drugs, topoisomerase inhibitors and antimicrotubular substances, for instance, affects both cancerous and normal cells by selectively targeting DNA replication and cell division.[20-22] Thus, creating new anticancer medications with reduced side effects is necessary. Therapeutic agents and therapeutic prospects, including drugs for cancer treatment, have always been found in medicinal plants. Vincristine, vinblastine, and taxol are chemotherapeutic drugs with natural origins or derivatives approved by the FDA.[23] Natural-based medicine contains pharmacologically active components obtained from biological or mineral-based sources and is utilized to diagnose, treat, mitigate and prevent disease.[24]

These studies showed the potential anticancer properties of *P. zeylanicum* Bulbs Ethanol (PZBE) extract. However, the molecular mechanism underlying PZBE extract-induced cancer cell death has not yet been investigated. Therefore, determining the anticancer efficacy of PZBE extract against the current work aimed to examine apoptosis and cell cycle analysis in Human cervical cancer (HeLa), colon cancer (HT-29), lung cancer (A549), and human breast cancer (MCF-7) *in vitro*.

MATERIALS AND METHODS

Collection of plant material

P. zeylanicum (Amaryllidaceae) plant bulbs were collected in July 2022 from Chettipalayam, Coimbatore, Tamil Nadu and India. A herbarium sheet was created and authenticated at the Botanical Survey of India, Southern Region, Coimbatore (Reference No. BSI/SRC/5/23/2022Tech/317). Collected botanical survey of India in Coimbatore preserved the specimens of the plants and properly documented them for use in the future.

Plant extract preparation

100 g *P. zeylanicum* dried bulb powder was extracted using the Soxhlet method using ethanol. Following extraction, vacuum solvent evaporator was used to evaporate the solvent. The extract was then kept at 4ºC to facilitate further usage.

Cell line culture

Breast cancer (MCF-7), colon cancer (HT-29) and lung cancer (A549) are all human cancers, and cervical cancer (HeLa) was obtained from Sri Vidya Mandir Arts and Science College in Uthagarai, Krishnagiri. Tissue Culture (TC) plates with 96 wells, the cells were grown in Dulbecco's Minimum Essential Medium (DMEM) with Trypsin-Phosphate-Versene Glucose (TPVG) solution, 10% Foetal Bovine Serum (FBS), 100 U/mL of Penicillin (Gibco-Invitrogen) and 100 g/mL of Streptomycin (Gibco-Invitrogen). The cells were subcultured every 3-4 days while being incubated in a CO_2 incubator (made by Haier Electric Co., Ltd.,) at 37ºC in a moistened environment containing 95% CO_2.

In vitro cytotoxicity

Most cytotoxicity tests have relied on cells' ability to resist an adverse. The test implies that dead cells or their derivatives do not reduce tetrazolium. The assay is affected by the number of cells present in addition to the mitochondrial activity per cell. The cleavage of MTT by living cells to a blue formazan derivative is a highly effective procedure on which the assay is developed. The principle at work consists of the disintegration of the tetrazolium salt MTT by the mitochondrial enzyme succinate dehydrogenase into a blue-colored reagent (formazan). The number of cells discovered to be proportionate to the amount of formazan produced by the cells used.

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle which the assay is based on. The principle involved is the cleavage of tetrazolium salt MTT into a blue-coloured product (formazan) bymitochondrial enzyme succinate dehydrogenase.

The DMEM medium containing 10% FBS was used to adjust the cell count to 1.0x105 cells/mL followed by centrifuge. 100 µL of the diluted cell solution (about 10,000 cells/well) was added to each well of a 96-well flat-bottom microtiter plate. When the cell population was confirmed to be adequate after 24 hr, the cells were centrifuged, and the pellets were suspended in 100 µL of different test samples the amounts prepared using maintenance media. The plates were subsequently kept at 37ºC in a 5% CO_2 atmosphere for 48 hr, with microscopic examination and observations recorded every 24 hr. The tested solutions were centrifuged after 48 hr and the pellets were re-suspended in 20 µL of MTT (2 mg/mL) in a minimum basic medium (MEM-PR (MEM without phenol red). The glass plates as well as incubated at 37ºC in a 5% CO_2 atmosphere for 2 hr. After adding 100 L of DMSO, the plates were lightly shaken to dissolve the newly formed formazan. At 540 nm, the amount of absorption was determined using a microplate

reader. The percentage cell viability was estimated using the formula below and the concentrations of drug or test samples required to suppress cell growth by 50% were calculated using the dose-response graphs. The method described below was used for calculating the percentage of growth inhibition.

$$\% \text{ cell viability } = \frac{\text{Mean OD of each test group}}{\text{Mean OD of control group}} \times 100$$

Cell cycle analysis

The cell cycle is the mechanism that controls the timing and frequency of DNA duplication and cell division. Cell cycle analysis was performed using flow cytometry as per the procedure given by Krishan (1975). The reagents Sodium citrate - 100 mg, Propidium iodide (50 µg/mL)-2.5 mg, RNase-4 mg and Tween-20-30 µL were dissolved in distilled water and formed up to 100 mL, which can be kept at 4°C for 6 months. Cells were trypsinized and centrifuged after their treatment with PZBE extract. The cells were subsequently stained for 30 min at room temperature in the dark with 1 mL of the prepared PI reagent combinations. Following incubation, the cells were flow cytometrically analyzed for populations at the sub-G0, G0/G1, S and G2/M phases of the cell cycle. FACSuite software (BD Bioscience, USA) was used to analyze the results.

Flow cytometer study of Annexin V/FITC-PI apoptosis staining to detect cell death

FITC Annexin V staining proceeds before the loss of membrane integrity that occurs during the last stages of cell death caused by either apoptotic or necrotic processes. As a result, FITC Annexin V labeling is generally employed in conjunction with a vital dye such as Propidium Iodide (PI) to allow the investigator to distinguish between early apoptotic cells, late apoptotic cells, and necrotic cells. Viable cells with membranes that are intact prevent PI, but dead and injured cells' membranes are permeable to PI. Dissolve 1 part of the 10X Annexin V binding buffer with 9 parts of distilled water to make a 1X working solution. The cells were trypsinized and centrifuged for 10 min at 5000 rpm before discarding the supernatant. Shake the pellet vigorously after adding 100 µL of 1X binding buffer. The cells were followed by staining for 15 min at room temperature in the dark with 5 µL Annexin V/FITC and 5 L propidium iodide. After the incubation period, 400 µL binding buffer was added and thoroughly mixed. Using a BD FACSverse flow cytometer, then observed the cells.

Statistical study

Software Package for the Social Science (SPSS) version 15.00 was used to conduct the statistical analysis; a one-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were also used. For the examination of the percentage of inhibition of cell growth, P values below 0.05 were considered significant and the data were expressed as Mean Standard Deviation.

RESULTS

Cell viability effect of PZBE extract

Table 1 and Figure 1 summarise the results of the MTT assay using PZBE extract on various cancer cell lines. The cell viability effect was evaluated at different concentrations of the extract (31, 25, 6, 25, 125, 250, 500, and 1000 µg/mL). A concentration-dependent activity was observed in all cancer cell lines. The PZBE extract showed 23.62, 18.40, 30.70, and 19.77 percent of cell viability in HeLa, A-549, MCF-7, and HT-29 cancer cells respectively at higher concentrations. At a 500 µg/mL concentration, the four cell lines demonstrated cell death with 36.45% in HeLa, 27.71% in A-549, 37.58% in MCF-7, and 30.02% in HT-29 cells. PZBE extract had IC_{50} values of 209.28 µg/mL for HeLa, 133.09 µg/mL for A-549, 264.46 µg/mL for MCF-7, and 264.75 µg/mL for HT-29 cell lines in the MTT assay (Table 2 and Figure 2). A-549 cell line demonstrated the lowest IC_{50} value among the four cell lines.

Evaluation of cell cycle analysis of A-549 cells by flow cytometry

To evaluate the growth stages of cells dealing with PZBE extract compared to the control group, cell cycle analysis was performed

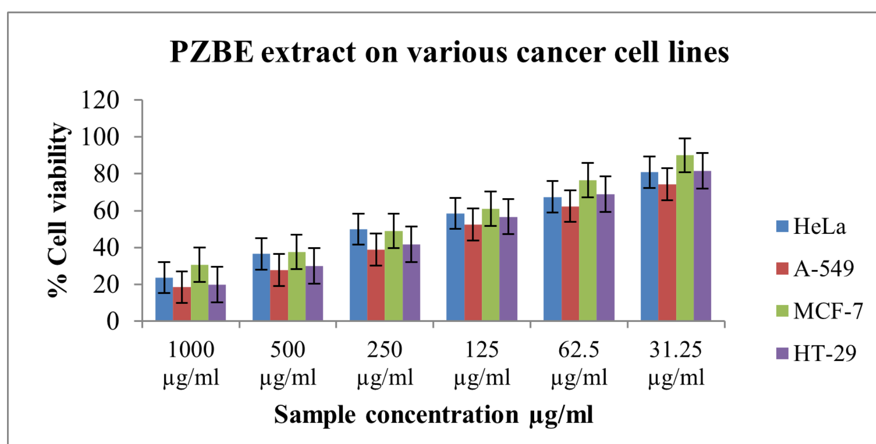


Figure 1: Cell viability effects of PZBE extract on various cancer cell lines.

on the *in situ* staining of A-549 lung cancer cells with propidium iodide-A. Figure 3 shows the DNA histograms and percentage of cell cycle stages of control and IC_{50} concentration 133 µg/mL of PZBE-treated A-549 cells after 72 hr incubation. The result demonstrates that most of the control cells are in G2-M stage (71.99%) of the cell cycle followed by S (17.46%) and G0-G1 (10.55%) stages (Figure 4). Whereas, the PZBE-treated A-549 cells show 42.12% of cells are in G0-G1 stage and 47.78% of cells are in G2-M phase. This indicates that the PZBE extract effectively arrests the A-549 cancer cells at early (G0-G1) and late (G2-M) stages of the cell cycle (Figure 4) when compared to control.

Flow cytometry was used to evaluate apoptosis in the A-549 cell line

Following a 72 hr treatment of A-549 cells with IC_{50} concentration 133 µg/mL PZBE extract, The Annexin-V FITC/propidium iodide-A double labeling experiment helped individual cells differentiate into living, early apoptotic, late apoptotic, and necrotic quadrants. Figure 5 displays the dot plot distribution in quadrants for A-549 cells, which were treated with PZBE extract and control cells. In A-549 cells treated with PZBE extract IC_{50} concentration, Double-staining of Annexin V FITC/PI demonstrated the effectiveness of PZBE extract in inducing apoptosis, with a rise an increase in the number of cells that exhibit early apoptosis (0.81), late apoptotic cells (67.65) and necrosis (7.62) compared to untreated cells (Figure 6). On the other hand, the live cells were highly (99.92%) found in control cells which is less in PZBE-treated cells (28.35%) (Figure 5). Additionally, the PZBE extract showed in the late apoptosis stage, the percentage of cells distributed was greater.

DISCUSSION

There are millions of people affected each year by cancer, which is one of the most prevalent diseases. According to estimates, cancer is the second biggest fatality of people. The American Cancer Society reported that cancer-related mortality makes up between 2 and 3% of all annual deaths reported worldwide.[25] Cancer is treated with several chemopreventive medicines, but many of them are toxic, limiting their full usage in therapies.[26] To tackle this condition, there has been an ongoing search for new anticancer medications for the past ten years. According to Richardson PG, *et al*,[27] more than 60% of currently utilized anticancer drugs come from natural sources. The biological actions of a large variety of natural compounds utilized as medicinal remedies are supported by scientific research. However, very little information was provided in the form of statistics or data on the potential toxicity that therapeutic plants may cause to people[28] However, cytotoxicity is a more reliable method for the analysis of the toxicity of test samples to the cancer cells in *in vitro* method.

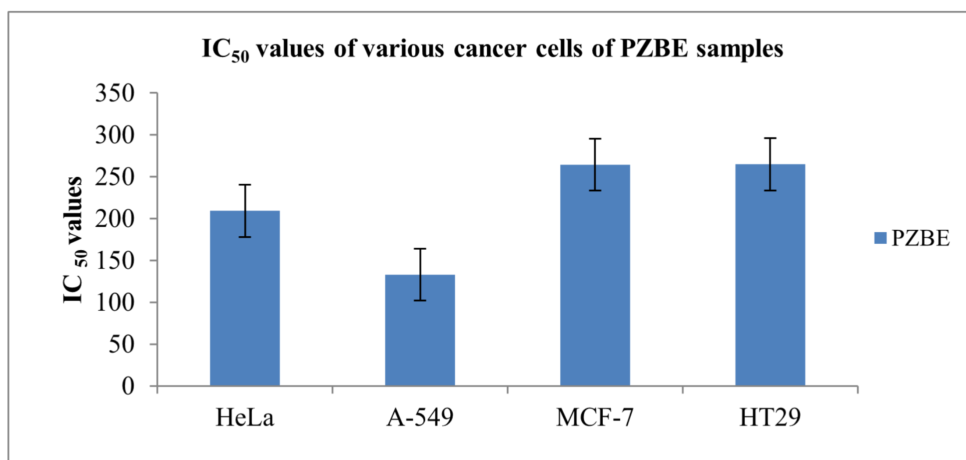


Figure 2: IC_{50} values of various cancer cells of PZBE samples.

Table 1: Cell viability effects of PZBE are extracted on various cancer cell lines.

Sl. No.	Concentration	Percentage of cell viability			
		HeLa	A-549	MCF-7	HT-29
1	1000 µg/mL	23.62±2.06^{b}	18.40±2.13^{a}	30.70±1.65^{c}	19.77±1.59^{a}
2	500 µg/mL	36.45±1.10^{b}	27.71±1.60^{a}	37.58±1.83^{b}	30.02±1.78^{a}
3	250 µg/mL	49.92±1.88^{b}	38.83±1.66^{a}	48.79±1.75^{b}	41.52±1.29^{a}
4	125 µg/mL	58.42±1.22^{b}	52.40±1.96^{a}	60.95±1.80^{c}	56.68±1.55^{b}
5	62.5 µg/mL	67.40±0.86^{b}	62.38±1.18^{a}	76.43±1.29^{c}	68.89±1.33^{b}
6	31.25 µg/mL	80.74±1.59^{b}	74.37±1.92^{a}	89.92±1.77^{c}	81.66±1.48^{b}

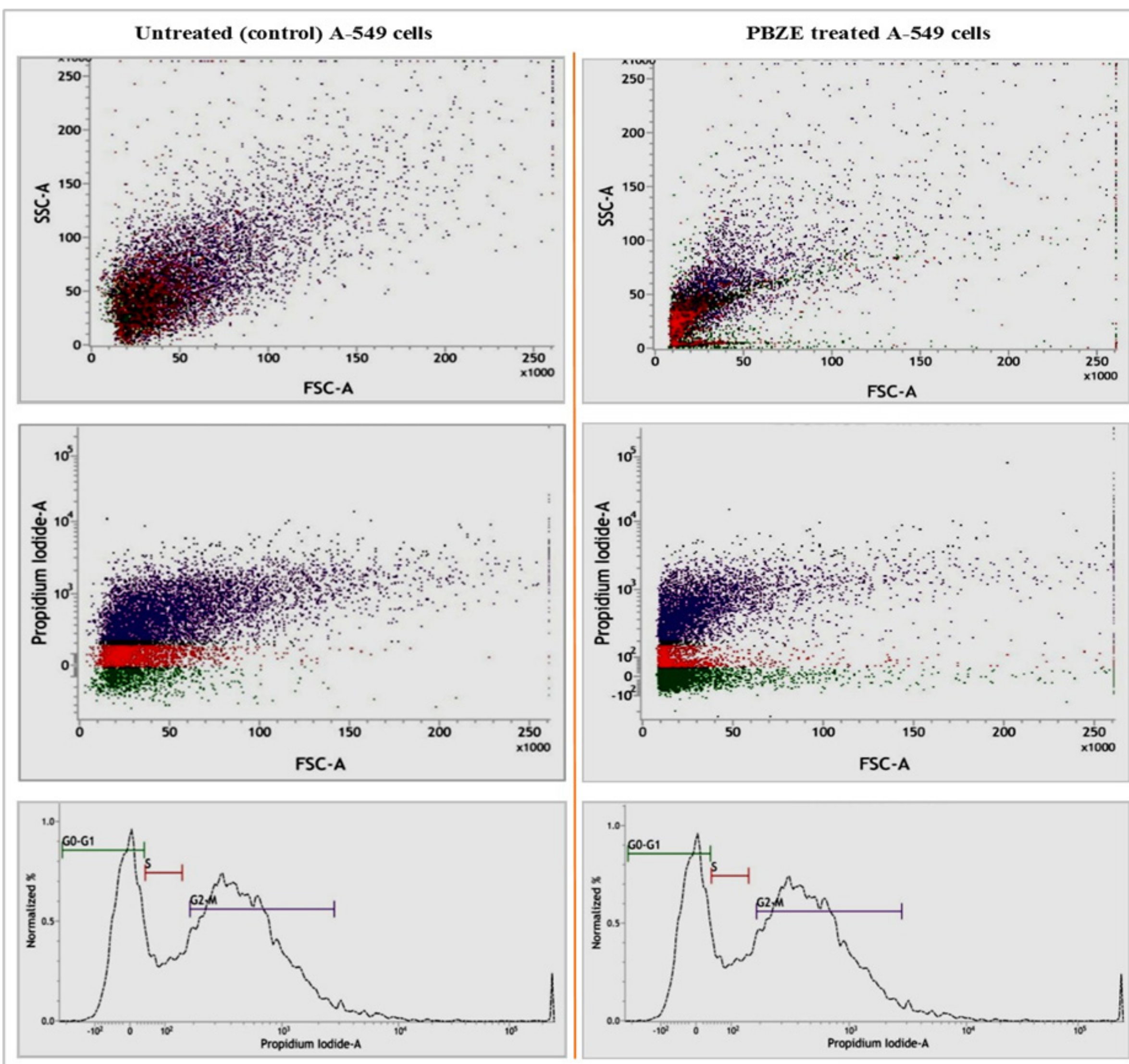
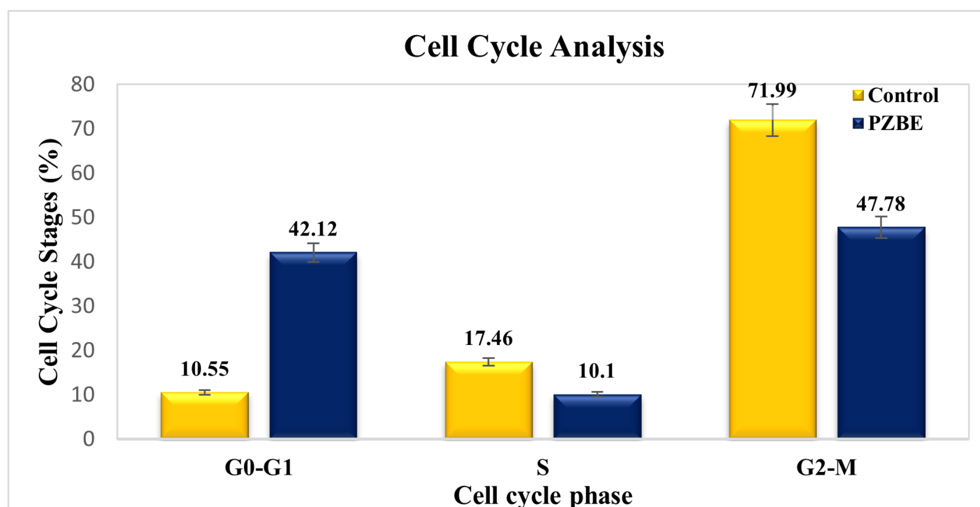


Figure 3: PZBE extract induces cell cycle arrest in the A-549 cell lines.

In the current study, the cytotoxicity associated with PZBE and extracts of *P. zeylanicum* showed different degrees of anticancer activity in four different cancer cell lines from humans (A-549, HeLa, MCF-7, and HT-29) by employing an MTT test. The higher concentration of the extracts exhibited lower cell viability, which indicated that PZBE extracts have a higher anticancer effect in higher doses. The PZBE extract showed 18.40 to 30.70% cell viability in the studied cancer cells. The PZBE extract had a greater cell viability effect. According to the IC_{50} values, PZBE extract controlled the viability of HeLa and A549 cancer cells significantly. On the other hand, the viability of HeLa and A-549 cancer cells was better controlled by PZBE extract. PZBE extracts are the most effective agents in the suppression of A-549 cells (IC_{50} value 133.09) When compared with different carcinoma cell cultures. Therefore, Cell death and cell cycle research were conducted only on the A-549 cell line treated with PZBE extract.

Cancer cells frequently have altered cell cycle regulatory signalling pathways and frequently lose their checkpoint systems, which promotes cell proliferation. To improve cytotoxic therapy development and overcome resistance to multiple drugs, it is thought that targeting ineffective cell checkpoints and apoptosis are potential tactics. [29,30] One potential tactic for reducing tumor growth is thought to be controlling the progression of the cancer cell cycle. Additionally, natural phytochemicals have been shown to improve immunological function, prevent the creation of DNA adducts or DNA intercalation, reduce angiogenesis and regulate hormone metabolism.[31] The current study assessed the percentage of cells at various stages of development to determine whether *P. zeylanicum* Bulb Ethanol (PZBE) extract might influence cell cycle progression. The cell cycle analysis findings reveal that the PZBE extract effectively arrested the proliferation of A-549 cells in both the cell cycle is divided into two phases: early (G0-G1) and late (G2-M). The PZBE extract-treated G0-G1



Values are denoted as mean±standard deviation.

Figure 4: Effects of the PZBE extract on the flow cytometric cell cycle analysis of A-549 cells using Propidium Iodide staining.

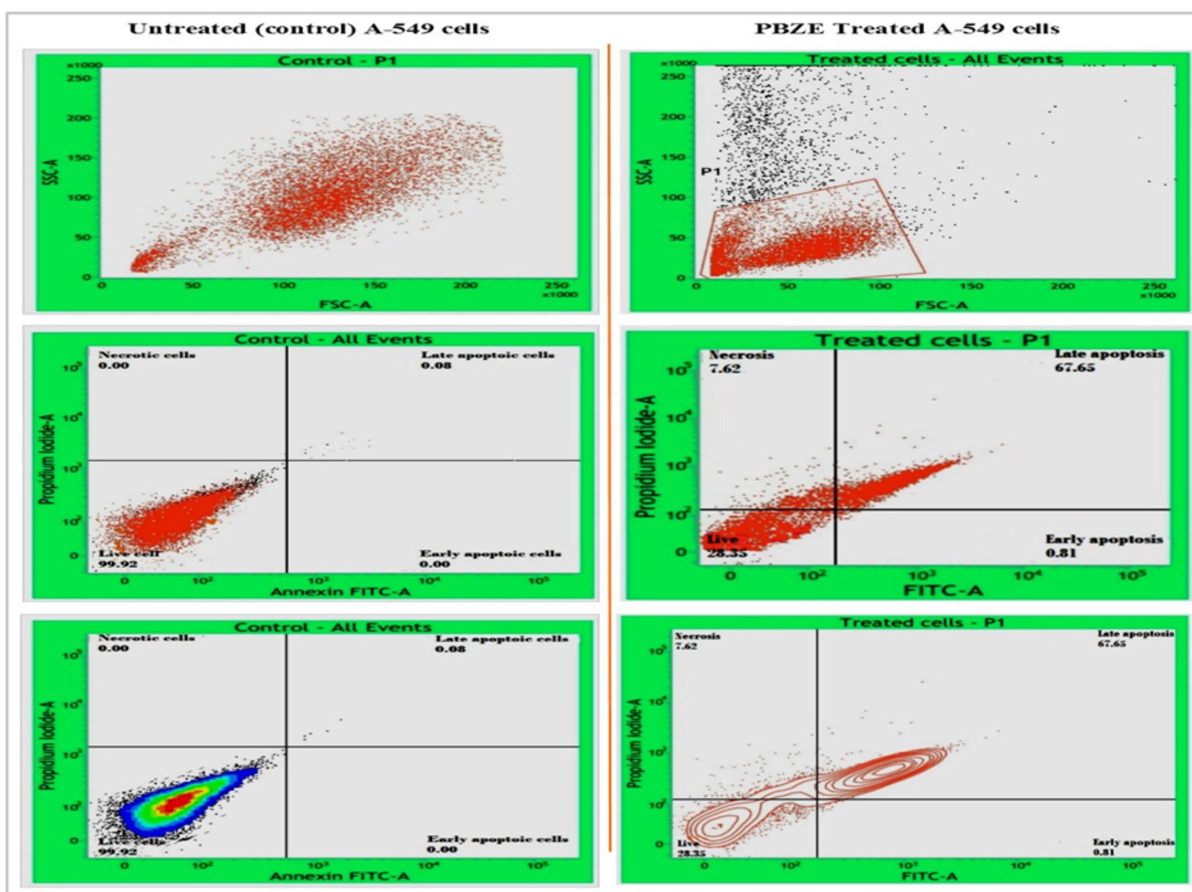
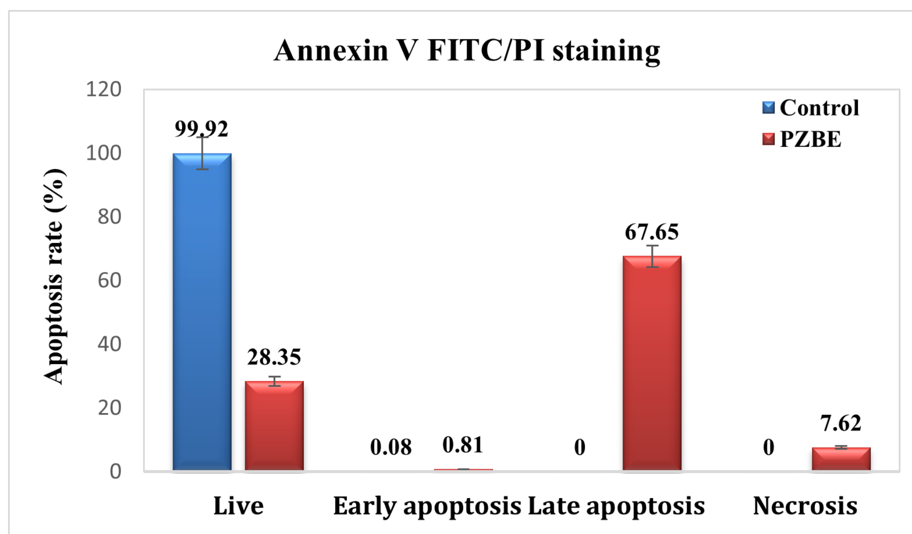


Figure 5: Dot plots for flow cytometry analysis of the apoptosis effects of A-549 cell lines treated with PZBE extract

phase cells significantly arrest the A-549 cell growth compared to control cells.

A series of biochemical processes known as apoptosis are responsible for regulating a wide range of physiological and pathological functions. The processes of apoptosis are repressed in drug-resistant cancer cells, which leads to an imbalance between cell death and growth.[32] Unique morphologic characteristics distinguish apoptosis. To identify apoptosis, a protein called annexin V is coupled to a green, fluorescent dye. Propidium iodide, more commonly known as (PI), is a red fluorescent dye that can stain the DNA of necrotic and late apoptotic cells with divided membranes.[33]



Values are denoted as mean±standard deviation.

Figure 6: Percentage of MCF-7 Apoptotic cells as determined by Annexin V-FITC and Propidium Iodide dual staining.

Table 2: IC_{50} **values of various cancer cells of PZBE samples.**

Sl. No.	Cell line	PZBE
1	HeLa	209.28±1.13^a
2	A-549	133.09±1.64^a
3	MCF-7	264.46±1.58^b
4	HT29	264.75±1.42^b

The Annexin V FITC/PI double-staining assay of the current investigation validated the PZBE extract's ability to effectively induce late apoptosis followed by necrosis and early apoptosis in A549 cells. The results were compared with the control group. The live A549 cell percentage was very high in untreated cells compared to PZBE extract-treated cells.

Members of the Amaryllidaceae family, including species of *Pancratium* genus, were examined for cytotoxicity, cell cycle analysis, and apoptosis induction. Different *Allium* extracts, both aqueous and alcoholic, were, previously explored in several cancer lines both as an individual treatment or in conjunction with cancer therapeutic agents, including lung,breast, cervical and colon cancer.[34-38] According to Prakash J, *et al*,[39] the methanol extract of *Zephyranthes citrina* (Amaryllidaceae) has demonstrated effective cytotoxicity activity against Cells from cervical cancer (Hela) and breast cancer (MCF-7) were tested. Previous studies on the anti-proliferative effects of *P.maritimum* stem, flower, bulb, and fruit extracts on seven cancer cell lines, including MCF-7, A549, and HeLa cell lines, have been published.[40,41] The current cytotoxic effect of the *P. zeylanicum* bulb ethanol extract is consistent with the Amaryllidaceae family's previous research findings.

According to Rezaei PF, *et al*,[42] and, Yuan SY, *et al*,[43] the cell cycle mechanism causing this arrest may interfere with DNA synthesis pathways or prevent the creation of the mitotic spindle. A-549 cancer cells' cycle was reported to be stopped by *P. maritimum* extracts at the S and G2/M stages. One beneficial property of anti-cancer medications is the selective inhibition of malignant cells in particular cell cycle phases.[44] As a result, the cell cycle arrest is consistent with *P. maritimum*'s earlier findings. According to recent research, Amaryllidaceae alkaloids may cause cancer cells to die by activating the apoptotic pathway.[45] In the annexin-V assay,[46] found the cancer cell death by apoptosis using various compound fractions. Furthermore, pancratistatin alkaloids induce apoptosis by targeting mitochondria in cancer cells.[47] However, the current apoptosis study results are similar to earlier reports Amaryllidaceae alkaloids and it indicate thatthe PZBE extracts is a powerful lead with potent apoptosis induction against A549 cells.

CONCLUSION

We conclude that the PZBE extract of *Pancratium zeylanicum* bulbs exhibited significant cytotoxic activity against several cancer cell lines. The MTT analysis was used to assess the PZBE extract with Annexin V as the positive control chemical based on four human cancer cell line, including HeLa, HT-29, A549 and MCF-7, with IC_{50} values of 209.28 µg/mL for HeLa, 133.09 µg/mL for A-549, 264.46 µg/mL for MCF-7 and 264.75 µg/mL for HT-29. The cytotoxic effects were primarily mediated by the induction of cell cycle and apoptosis pathways. When compared with the control, the results that have been identified in G2-M and G0-G1 cells by 42% and 44.78%, respectively. In A-549 cells, the PZBE extract significantly induced G0-G1 arrest. Furthermore, the PZBE extract effectively caused apoptosis in A-549 cells with the IC_{50} concentration, The Annexin-V double-staining experiment revealed an increase in the percentage of early and late apoptotic

cells compared with untreated cells. We are certain that the PZBE extract could be a source for the isolation of anticancer molecules as a result.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PZBE: Pancratium zeylanicum Bulb Ethanol; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **HeLa:** Human Cervical Cancer; **HT-29:** Human Colon Cancer; **A549:** Human Lung Cancer; **MCF-7:** Human Breast Cancer; **DMEM:** Dulbecco's Minimum Essential Medium; **TPVG:** Trypsin-Phosphate-Versene Glucose; **FBS:** Fetal Bovine Serum; **AChE:** Acetyl Cholinesterase; **WHO:** World Health Organization; **FDA:** Food and Drug Administration; **DNA:** Deoxyribonucleic Acid; **PI:** Propidium Iodide; **FITC:** Fluorescein Isothiocyanate; **SPSS:** Statistical Package for the Social Science; **ANOVA:** Analysis of Variance; **DMRT:** Duncan's Multiple Range Test.

SUMMARY

The anti-cancer properties of *P. zeylanicum* bulbs were studied against the most prevalent and deadly cancers. In the inhibition of HeLa, A-549, and MCF-7 cell lines, the PZBE extract exhibited a stronger viability of cells response. As a result, ethanolic extracts constituted superior anticancer drugs. Furthermore, as compared to other cell lines, ethanolic extracts revealed the lowest IC_{50} values in the research of A-549 cell line suppression. PZBE extracts significantly prevented the cell cycle regulatory pathways at the early (G0-G1) and late (G2-M) phases, based on cell cycle studies of PZBE extract-treated A-549 cells. The apoptosis investigation employing the Annexin V FITC/PI double-staining assay revealed the results that the PZBE extract exhibited the most potent late-stage apoptosis in the induction process, followed by necrosis. Nevertheless, results from *in vitro* biological activity investigations of *P. zeylanicum* indicated the fact that the plant's ethanol extract is the most therapeutic powerful.

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Cite this article: Priya ES, Sheela DS, Gnanadeebam DS, Raja BD, Viswanathan P. Screening of *in vitro* Anticancer Activity of Various Human Cancer Cell Lines and Induced Apoptosis and Cell Arrest by Ethanolic Bulb Extract of *Pancratium zeylanicum*. Pharmacog Res. 2024;16(4):718-26.