

Original Research Article

In vitro apoptosis-inducing effect of pinostrobin on MCF-7 breast cancer cells

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Abstract

Purpose: To investigate the ability of pinostrobin to instigate apoptosis in human adenocarcinoma cell line (MCF-7).

Methods: To visualize the distinct features of apoptosis, cells were subjected to parallel staining with AO/PI dual fluorescent staining, while Annexin V-FITC flow cytometry facilitated the evaluation of the extent of death triggered by pinostrobin.

Results: Unique morphological changes, such as nuclear condensation and fragmentation, cell shrinkage, membrane loss, and blebbing, characteristic of apoptosis, were evident following treatment with pinostrobin at concentrations of 0, 3, 10, and 30 μ M. The percentage of cell survival drastically reduced from 97.78, 93.38, and 3.87 % to 0.83 % after exposure to 3, 10, and 30 μ M concentrations of pinostrobin, respectively. As the viability drastically reduced, a greater percentage of cancer cells shifted to the late apoptotic stage in a concentration-dependent manner.

Conclusion: This study demonstrates the potential of pinostrobin to kill breast cancer cells by forcing them to surrender their growth potential. Further analysis is recommended to ascertain this apoptotic mechanism.

Keywords: Apoptosis, Breast cancer, Pinostrobin, Annexin V-FITC flow cytometric assay, Natural product

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INTRODUCTION

Apoptosis is a highly regulated process that involves a cascade of biochemical and structural changes, ultimately leading to cell death [1]. It is crucial in the maintenance of cellular homeostasis and organismal development. Apoptosis is mediated by caspases [2] and is triggered by various stimuli, including DNA damage [3], oxidative stress [4] and death receptor activation. It is a double-edged sword, playing a vital role in both health and disease, and it occurs either through the intrinsic or

extrinsic pathways [5], which are interconnected, allowing for a coordinated response to various apoptotic signals. Apoptosis serves as an impediment to the uncontrolled proliferation of cancer cells [6]. Unfortunately, cancer cells evade apoptosis in their bid to proliferate uncontrollably and strengthen their tumor cell population. They achieve this by developing strategies like obliterating the function of the tumor suppressor protein, TP53. Alternatively, cancer cells also evade apoptosis by enhancing the expression of Bcl-2 and Bcl-xL survival signal (Igf1/2), upregulation of Bax, Bim, and Puma,

accompanied by the modulation of the ligand-activated extrinsic pathway [7]. In most cancer cases, mutations are observed in the apoptotic pathways, some of which are acquired. Cancer cells may exhibit epigenetic modifications, most notably DNA methylation or histone modifications that silence proapoptotic genes. The consequence of apoptosis dysregulation is to drive cancer cells' viability, promote metastasis, increase genome instability and resist therapy. Breast carcinoma is still a societal health concern as cancer cases increase. The lifetime incidence of breast cancer among the female population in the United States is estimated to be around 12% with an estimated death of 42,170 in 2025 [8]. The rate of breast carcinoma in the Nigerian population is increasing because of urbanization and lifestyle changes [9]. Resistance to therapy and undue side effects following treatment have necessitated the search for more therapeutic options from nature. Phytochemicals are templates for the development of novel drugs and have been reported to have less toxic side effects [10] and selectivity towards cancer cells. Evidence from epidemiological studies corroborates increased consumption of phytochemical-rich fruits and vegetables, leading to a reduced potential for the disease [11,12]. Pinostrobin, 5-hydroxy-7-methoxy flavanone, is a natural flavanone with anti-inflammatory, antibacterial, anti-spasmodic and antimutagenic properties. It possesses good pharmacokinetic properties and has a strong affinity towards estrogen receptor [13]. This study investigates the apoptosis-modulating potential of pinostrobin in breast cancer cells using morphological assessment and Annexin V-FITC flow cytometry.

EXPERIMENTAL

Extraction and isolation of pinostrobin

Pinostrobin was extracted from the rhizomes of *Alpinia mutica*, which were sourced from the Forest Research Institute Malaysia (FRIM). The rhizomes were sliced, spread out at ambient temperature and finely ground before use. The extraction and isolation processes were carried out as earlier reported [14].

Cell culture

MCF-7 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium supplemented with 10% FBS and 1 % penicillin–streptomycin under aseptic conditions in a Class II biosafety cabinet following GCCP guidelines [10]. Cells were incubated at 37 °C under humidified conditions with 5 % CO₂. At ~80 % confluency,

cells were detached with trypsin-EDTA, neutralized with complete medium, and centrifuged at 1000 rpm for 5 min. Viable cells were counted using a hemocytometer and subcultured ($0.5 - 1 \times 10^6$ cells) into a new 25 cm² flask at a 1:4 split ratio.

Fluorochrome-based morphological analysis of apoptotic cells

An AO/PI dual-staining assay was conducted to evaluate pinostrobin-induced cell death in MCF-7 cells. 3×10^5 cells were plated and incubated overnight to allow adhesion before treatment with pinostrobin (3, 10, or 30 µM) for varying durations. The cells were collected following a 5-minute centrifugation at 2000 rpm, washed twice with ice-cold PBS, and resuspended in 20 µL PBS. The cell suspension was stained on ice with AO/PI solution (10 µg/mL each) and examined using a fluorescence microscope (Carl Zeiss Axioskop 2 plus). A minimum of 200 cells across three randomly selected fields were scored for cell survival, apoptosis, and necrosis. All experiments were carried out in triplicate.

Apoptosis analysis using Annexin V-FITC and PI staining

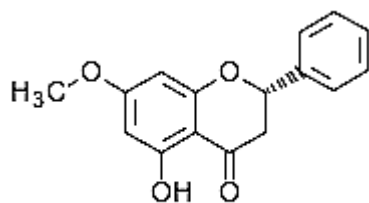
Apoptotic cells were detected using the Annexin V-FITC Kit (BD Pharmingen, San Diego, CA, USA). MCF-7 cells (1×10^5) were treated with pinostrobin (3, 10 and 30 µM) for 24 and 48 h with 1 % DMSO as control. Treated adherent and floating cells were prepared and resuspended in binding buffer for apoptosis analysis. Cells were stained with annexin V-FITC (5 µL) and propidium iodide (5 µL), incubated for 20 min at room temperature in the dark, and analyzed by flow cytometry (Cyan ADP, DAKO, Denmark) at 488 nm excitation and data were processed using Summit software (v4.3).

Statistical analysis

Data analysis was conducted in GraphPad Prism 5.0. (GraphPad Software Inc., La Jolla, CA, USA). Group differences were evaluated using One-way ANOVA followed by Tukey's post hoc test, with significance defined at $p < 0.05$.

RESULTS

The 2-dimensional structure of pinostrobin, with a molecular weight of 270.28, is shown below (Figure 1). As has been reported earlier, it possesses good pharmacokinetic properties and drug-likeness [13].



C16419

Figure 1: Pinostrobin ((2S)-5-hydroxy-7-methoxy-2-phenyl-2,3-dihydrochromen-4-one)

Morphological changes displayed by pinostrobin-treated MCF-7 breast cancer cells

During apoptosis, cells undergo distinct morphological changes that enable their recognition and removal by phagocytes. The

results revealed different phases of apoptosis as indicated in Figures 2 - 4. The nuclei of pinostrobin-treated MCF-7 cells become more compact and denser due to the condensation of the chromatin (Figure 2 B). Nuclear fragmentation with apoptotic bodies, which is a feature of early stages of apoptosis, was also observed (Figure 2). Later features of apoptosis became obvious, like membrane loss and blebbing (Figure 2 D). Few necrotic cells were seen with red fluorescence and an intact nuclear membrane. These necrotic cells became prominent at a higher treatment concentration following a 48-hour treatment (Figure 4). Different morphological changes were prominent after exposure of the breast cancer cells to pinostrobin (Figure 3 and Figure 4).

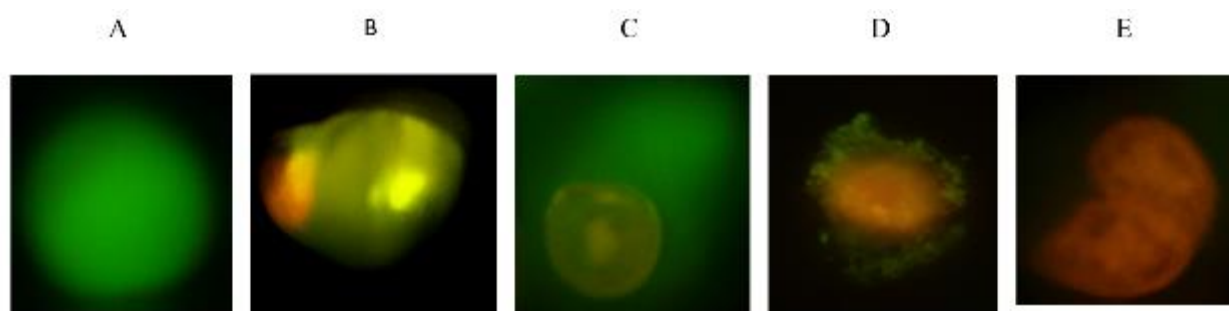


Figure 2: Fluorescence micrographs (400X) of HCT-116 cells after AO/PI staining. Representative micrographs showing: (A) viable cell with green fluorescence and intact nuclei, (B) initial phase of apoptosis shown with nuclear condensation, (C) late apoptotic cell with nuclear fragmentation and apoptotic bodies, (D) late apoptotic cell with membrane blebbing and loss of integrity, and (E) necrotic cell exhibiting red fluorescence with an intact nuclear membrane

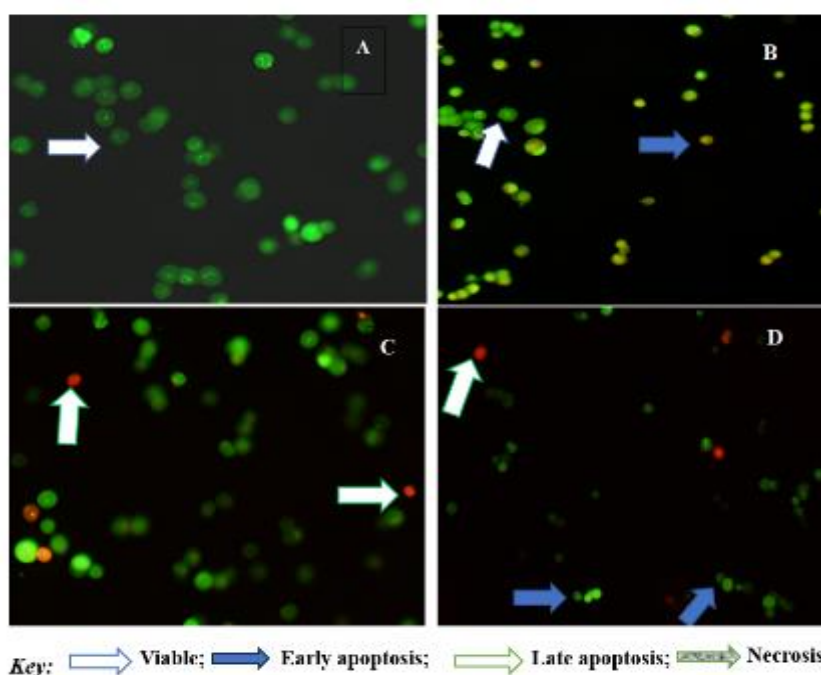


Figure 3: AO/PI-stained MCF-7 cells after 24 h pinostrobin treatment. (A) Control, (B) 3 μ M, (C) 10 μ M, and (D) 30 μ M. Cells that survived fluoresced green while apoptotic cells displayed nuclear condensation/fragmentation, and necrotic cells showed red fluorescence with disrupted nuclear membranes. Images at $\times 100$ magnification

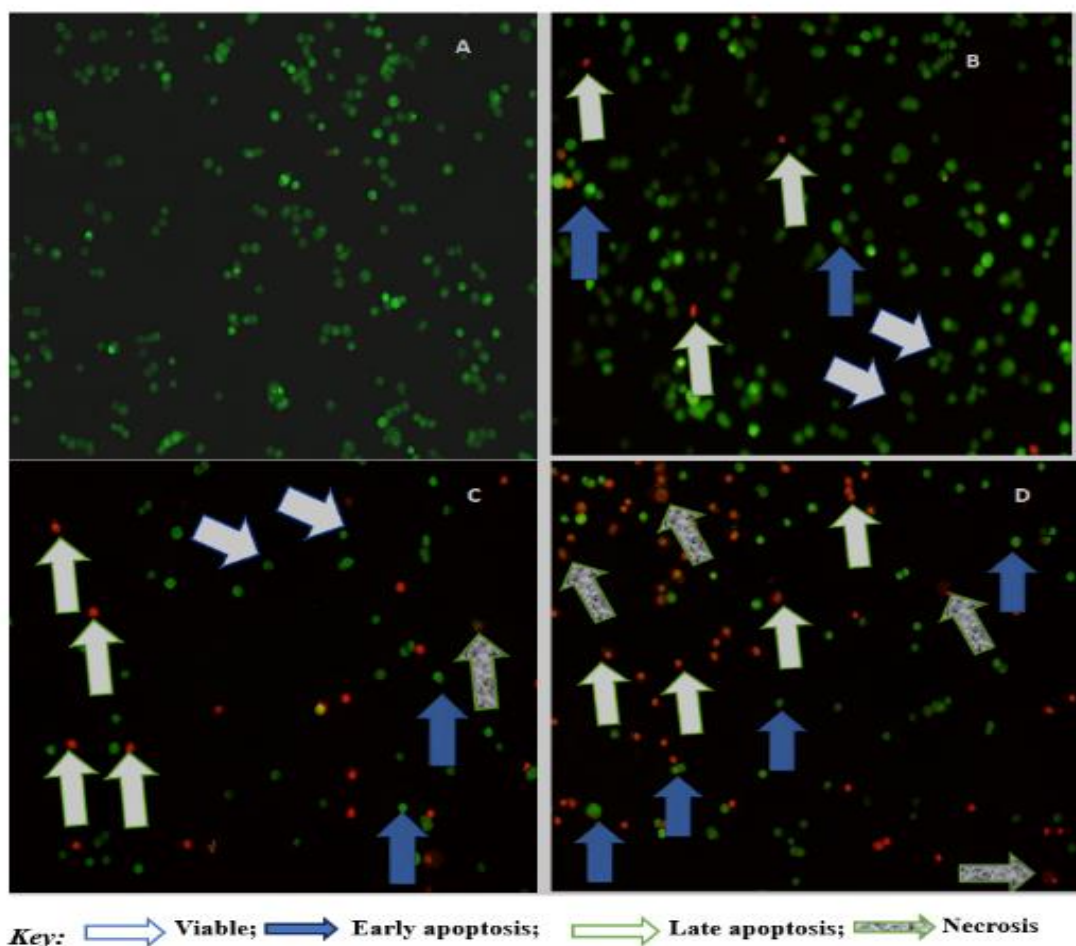


Figure 4: AO/PI staining of MCF-7 cells after 48 h pinostrobin treatment (3 – 30 μ M). Images show viable (green), apoptotic (bright green, condensed/fragmented nuclei), late apoptotic (red, fragmented nuclei), and necrotic cells (faint red, intact nuclei). $\times 100$ magnification

AO/PI staining highlights cellular alterations in MCF-7 cells

Pinostrobin triggers apoptotic cell death in breast cancer cells, increasing with both concentration and duration of exposure (Figure 4 C). After exposure to pinostrobin at different time points, unique morphological characteristics peculiar to apoptosis were observed in the cancer cells. The untreated cells had an intact membrane, while pinostrobin-treated cells had reduced cell volume, resulting in the shrinkage of the cancer cells. Chromatin condensation (Figure 4 C) was also observed. The condensed chromatin was marginated, forming crescent-shaped structures (Figure 4 C). Small, bubble-like protrusions and some membrane-bound apoptotic bodies were also observed after treatment with pinostrobin. These morphological changes were more evident and intense as the concentration of pinostrobin increased.

The number of cells in the different stages of apoptosis was quantified and observed to increase as a function of dose and exposure time (Figure 5).

Cell population in the lower left quadrant is viable, in lower right quadrant are cells at early apoptosis, in upper right quadrant at late stage of apoptosis, and in upper left corner at the necrotic stage. The numbers are the percentages of cell population in each quadrant. At 48 hours following the exposure of MCF-7 cells to different doses of pinostrobin, the percentage of late apoptotic cells increases drastically from 0.18 to 75.04 % in the control and 30 μ M-pinostrobin treated groups. There was also a simultaneous decrease in the percentages of viable cells (Figure 6).

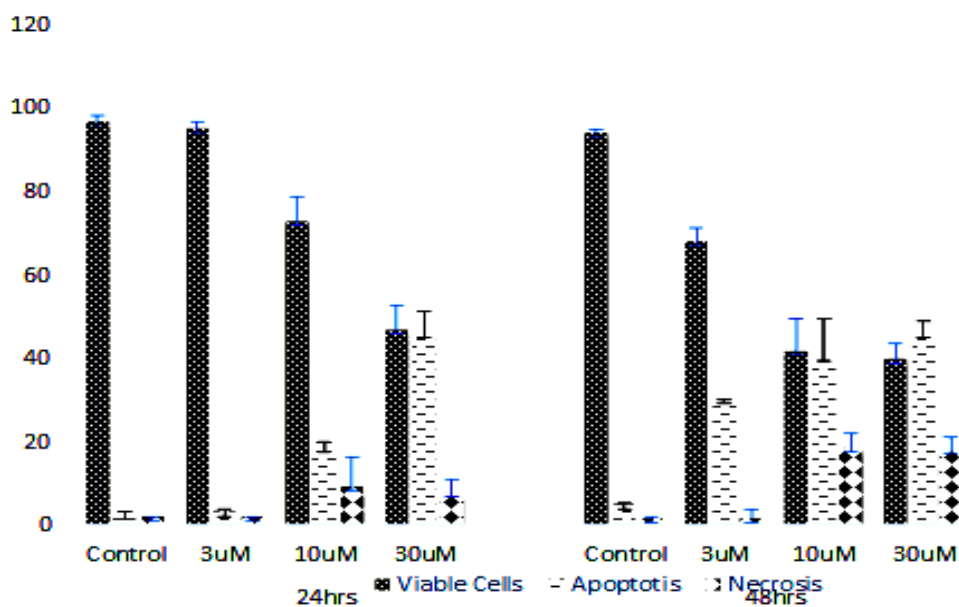


Figure 5: Quantification of AO/PI-stained MCF-7 cells following pinostrobin treatment. Data are presented as mean $\pm$ SD (n=3). $p < 0.05$ vs. control at corresponding time points

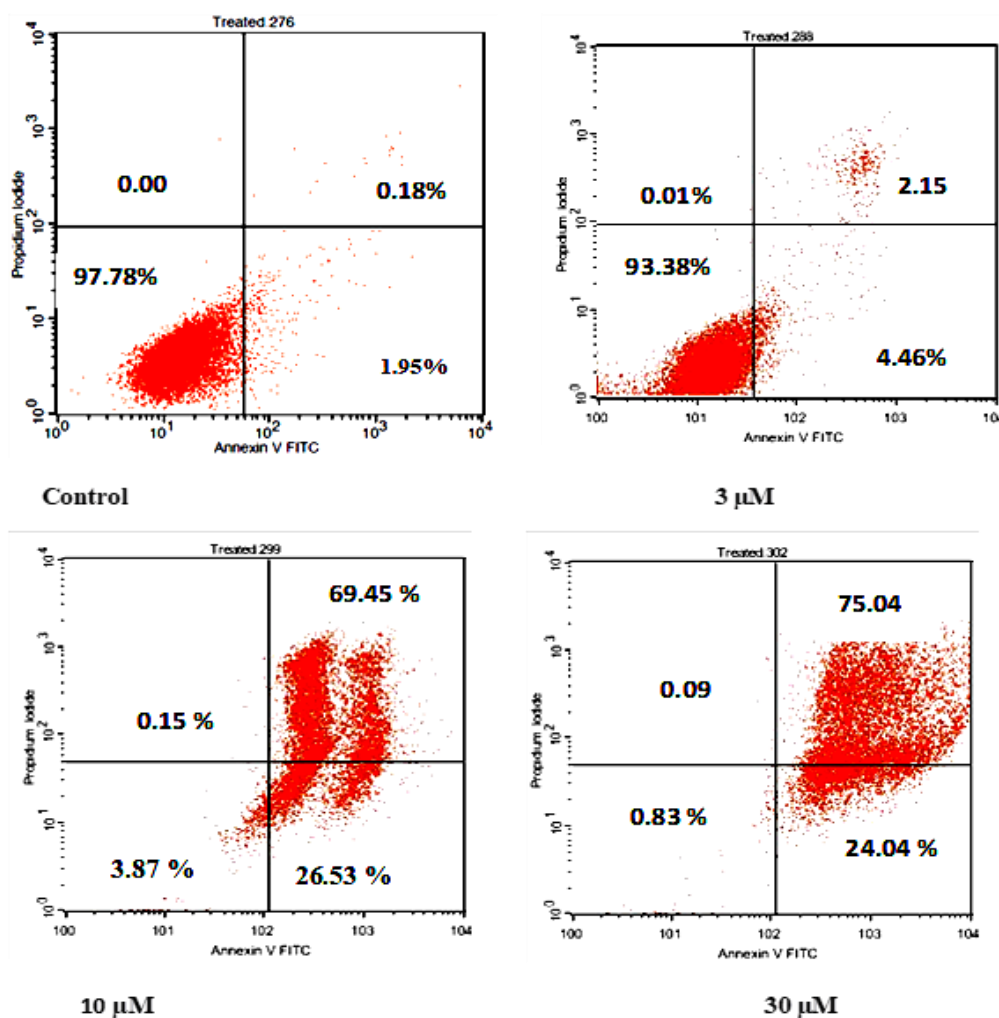


Figure 6: Annexin V histogram of MCF-7 cells after 48 h pinostrobin treatment

DISCUSSION

This study evaluated the apoptotic effects of pinostrobin in MCF-7 breast cancer cells. Following a time-dependent exposure of pinostrobin to breast cancer cells, the unique features of apoptosis were evident. These distinct morphological changes distinguish apoptosis, a programmed cell death, from necrosis, which arises from damage to the cell. Pinostrobin exhibited dose-related induction of early and late phases of apoptosis. Pinostrobin following 24- and 48-hour treatments. The early stages involve a decrease in cell volume due to ion flux and water balance. During this process, the nucleus of a cell becomes more compact and denser due to the condensation of the chromatin. This occurs in various cellular contexts, including nuclear shrinkage, nuclear condensation and cytoplasmic condensation. Consequently, controlling intrinsic apoptosis could benefit cancer treatment and prevention. Induction of apoptosis is a cherished approach and target for subverting the atrocities of breast cancer and other types of cancer [12]. The initial phase of apoptosis recruits phagocytes without inflammation, unlike late apoptosis and necrosis, which trigger inflammatory signals [15].

Notably, few necrotic cells were detected at the lower concentration and during the 24-hour exposure. This finding implies that cell death might have been due to nutrient depletion in the growth media or contact inhibition. From a previous report, it is evident that pinostrobin's ability to inhibit estrogen receptor resulted in the morphological changes evident in this study [13]. Similar to a report by Abaza *et al*, most flavonoids induce apoptosis by upregulating pro-apoptotic factors and inhibiting the anti-apoptotic proteins [16]. It is therefore speculated that the morphological changes observed in pinostrobin-treated breast cancer cells may be due to a similar mechanism of action [16,17].

The cell membrane's phospholipid distribution is asymmetric, with distinct phospholipids localized to specific leaflets: the outer leaflet has phosphatidylcholine and sphingomyelin predominantly, while phosphatidylserine is mainly located on the inner leaflet. This uneven distribution of molecules underpins key cellular functions, including signal transduction. And membrane structure. However, this asymmetry is often disrupted during apoptosis such that phosphatidylserine is externalised on the cell's plasma membrane. This disruption in the membrane phospholipid is a marker for apoptosis and is measurable by flow cytometry with fluorochrome-tagged Annexin V targeting

phosphatidylserine. After exposure to pinostrobin, there was a significant concentration-dependent transition of cancer cells from viability to late apoptosis at 48 h, suggesting an activation of apoptotic pathways ($p < 0.05$). These observations were similar to the apoptotic death observed with quercetin [18-19]. In this study, the apoptotic response of MCF-7 cells to pinostrobin increased proportionally with dose.

The mechanism behind this apoptosis induction is suspected to be partly due to its inhibition of the human estrogen receptor [13]. However, further investigation would be required to look into it.

CONCLUSION

Apoptosis represents a pivotal mechanism in cancer therapy, and agents capable of engaging this pathway remain of high interest in drug discovery. These findings demonstrate that pinostrobin, a compound previously described as drug-like with favorable pharmacokinetic characteristics, induces apoptotic death in breast cancer cells. While these results are encouraging, further mechanistic studies and *in vivo* evaluations are warranted to fully establish its therapeutic relevance and potential as a candidate for anticancer drug development.

DECLARATIONS

Acknowledgement/Funding

None.

Ethical approval

Not required.

Use of Artificial intelligence/Large language models

We declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities about claims relating to the content of this article will be borne by the authors. Conceptualization, methodology, I.E., and IU; formal analysis, investigation, IE; writing—original draft preparation, IE and IU. All authors have read and agreed to the manuscript.

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