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Extract Formulation of *Zingiber officinale* and *Phylanthus reticulatus* Induces Apoptosis of WiDr Colon Cancer Cells

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Abstract. People use ginger (*Zingiber officinale*) extensively for health purposes. Our previous studies have shown that *Phyllanthus reticulatus* has strong antioxidant capabilities. This study investigates the anticancer potential of ethanolic extract of *Z. officinale* and *P. reticulatus* formulations (ZP), focusing on their cytotoxicity and mechanisms against cancer cells. Samples were extracted using the maceration method. The method for determining the cytotoxicity of cells is MTT, the cell cycle is flow cytometry, apoptotic cells are flow cytometry and double staining, and caspase-8 and caspase-9 expression using the immunocytochemistry method. The analysis of caspase-8 and caspase-9 expression using the immunocytochemistry method. Among the tested formulations, the ethanolic extract of ZP_1:0 showed the most promise against WiDr colon cancer cells (IC_{50} value of 102.1 µg/ml). The ZP_1:0 demonstrated dose-dependent apoptosis induction in WiDr cells, showing early and late apoptosis, with minimal necrosis. The extract activates extrinsic and intrinsic apoptotic pathways in inducing cancer cell death. Compared to doxorubicin, the ZP 1:0 formulation selectively promotes apoptosis with reduced necrotic damage, suggesting the potential for reduced systemic toxicity. The study supports the potential of *Z. officinale* as a source of safer, plant-based anticancer agents, particularly for colon cancer, while recommending further research to identify and enhance active compounds.

Keywords: Caspase; cell cycle; cytotoxicity assay; *Phyllanthus reticulatus*; *Zingiber officinale.*

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INTRODUCTION

Cancer is the world's leading cause of death to now (Ali Salman, 2023; Shah et al., 2019; Sung et al., 2021; Zhou et al., 2024). This is a global challenge in dealing with this health disorder (Neil, 2023). By 2022, there will be 20 million new cases with 10 million deaths (Bhawalkar et al., 2024). So far, there are several types of cancer treatment efforts, including photodynamic therapy, photothermal therapy, cancer gene therapy, nanoparticle-drug therapy, and immunotherapies (Bidram et al., 2019; Schirrmacher, 2019). Some of these tend to be expensive and cause side effects, resulting in damage to the tumor-bearing host and the host's immune system (Schirrmacher, 2019) and growing worries concerning drug resistance (Abotaleb et al., 2020).

Traditional researchers have been focusing on examining the possible applications of plant extracts in the handling of human illnesses, such as cancer. There are now numerous plant species being employed to treat or prevent cancer (Mudyiwa et al., 2022) (Neneng et al., 2020). Plants come with an entourage of natural molecules, some of which may enhance or antagonize the anticancer effects of others. To overcome this entourage problem, a hunt for novel medicinal molecules that are responsible for the observed anticancer effects is necessary. Natural chemicals originating from plants are of great interest in the ongoing search for safer and more effective medicines (Abotaleb et al., 2020; Garcia-Oliveira et al., 2021). Numerous effective anticancer medications that are now being used in clinical settings and have shown a high degree of efficacy are made from plants (Majolo et al.,

2019). The fact that more than 60% of anticancer medications on the market today come from natural sources emphasizes the significance of plant-based bioactive chemicals in therapeutic development (Asma et al., 2022).

Ginger (*Zingiber officinale*) is a plant widely used by the community for health, such as antioxidant, immunoregulatory, microflora regulatory, intestinal protective, and anticancer properties (Alfuraydi et al., 2024; Mao et al., 2019; Nguyen et al., 2024; Sirui et al., 2024; Zhou et al., 2024). Nguyen et al., (2024) reported that the essential oil of *Zingiber officinale* has anticancer abilities against Hep3B, SK-LU-1, MCF-7, SK-LU-1, SW480, and HepG2 cancer cells. There are many bioactive compounds in *Z. officinale,* including phenolic compounds, terpenes, polysaccharides, lipids, organic acids, also fibers (Mao et al., 2019). Bioactive compounds of *Z. officinale* have been shown to have anticancer effects through various types of mechanisms, such as proliferation inhibition of cancer cells, apoptosis induction of cancer cells, inhibition of angiogenesis, dissemination, and migration of cancer cells (Zadorozhna & Mangieri, 2021). According to (Zadorozhna & Mangieri, 2021), the anticancer mechanism of 6-shogaol was on apoptosis of cancer cells via caspase pathways and cell cycle arrest in colon cancer.

Currently, many researchers are looking for various weed plants to be utilized for health, for both preventive and curative purposes. *Phyllanthus* species are widely found in tropical regions. *P. reticulatus* is commonly used by the public to treat hypertension, fever due to malaria, constipation, skin and urinary disorders, inflammation, urinary tract infections, rheumatism, and eye diseases (Jantan et al., 2019). *P. reticulatus* leaf and fruit extracts show immune-stimulator activity (Kumar et al., 2014), and analgesic and anti-inflammatory effects (Akhter et al., 2018). This species is reported to contain many secondary metabolites, including polyphenols, flavonoids, sterols, alkaloids, terpenes, and tannins (Jantan et al., 2019).

Many studies report ginger's anticancer properties and some anticancer abilities. However, so far, there have been no studies on the cytotoxic properties of the two formulations against cancer cells. An anticancer agent can act on the process of proliferation or the process of apoptosis. Further studies of the cytotoxic ability of *Z. officinale-P. reticulatus* formulations can be done through the study of specific effects on the proliferation or death/apoptosis of cancer cells. Exploration of the mechanism of cancer cell apoptosis through intrinsic or extrinsic mechanisms has deepened the understanding of the anticancer ability of *Z. officinale-P. reticulatus* formulations. The phytopharmaceutical ability of a plant is inseparable from the presence of bioactive compounds in the plant. Qualitative and quantitative filtering of bioactive compounds in ginger-mangsian formulations will enrich the information on the potential of the formulation as an anticancer agent.

This study provides scientific evidence of the anticancer activity of *Z. officinale-P. reticulatus* formulations against several cancer cell lines. This study aims to examine in vitro the anticancer activity of *Z. officinale-P. reticulatus* formulation and its mechanism against cancer cells that are most susceptible to the formulation. It provides scientific validity for the use of *Zingiber officinale–Phyllanthus reticulatus* formulations as alternative cancer therapies; this work has important scientific and societal implications. The results could advance the sustainable use of regional medicinal plants, enhance public health outcomes, and aid in the creation of natural, reasonably priced anticancer medicines for society, especially in areas with limited resources.

METHODS

This study was laboratory-based research. The cytotoxicity assay of the extract formulation (*Z. officinale-P. reticulatus (ZP*) at ratios of 1:0, 0:1, and 1:1 (Kristiani et al., 2024)) against three types of cancer cells (WiDr, MCF-7, and HeLa)). . The extract formulation, which showed an IC_{50} value of around 100 ppm, is called the potential extract formulation. Next, an anticancer mechanism test (cell cycle and apoptosis assay) was conducted on the potential formulation.

Sample extraction

Samples (Figure 1) of *Z. officinale* rhizomes were obtained from a local market in Semarang, while *P. Reticulatus* fruits were taken from gardens in the Tembalang area of Semarang. Samples were extracted using the maceration method with ethanol (Kristiani et al., 2024). The study was conducted in five replicates.

(A) (B)

Figure 1. *Z. officinale* rhizome (A) and *P. reticulatus* fruits (B)

In the maceration process, the mixture of dry sample: ethanol (1:1.5 w/v) was incubated for 24 hours and then filtered to extract the filtrate. The precipitate is re-macerated three times with the same process and time. The filtrate combination is concentrated using a Rotavapor R114 Buchi rotary evaporation operating under vacuum at 40°C (Eyela A-1000S).

A cytotoxic assay using the MTT method

The cytotoxicity assay used the [3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] (MTT) method (Bahuguna et al., 2017) with slight modifications. This study used sodium dodecyl sulfate (SDS) to dissolve the formazan crystals.. The 1 × 10^4 cells/well cell suspension was inserted into *a 96-well plate* incubated in a 5%CO_2 incubator at 37°C for 24hours. The concentration series samples were 62.5 - 1000µg/mL. Each test is always included with doxorubicin, cell control, and medium control. The 10µL of MTT 5mg/mL was added and incubated under the same conditions as before. After the incubation period, 100 µL of 10% stopper, SDS in 0.1N HCl. Next, *the plate* was wrapped and left overnight at room temperature. After this, the absorbance of each well was read on an ELISA *reader* (SLT 240 ATC) at a wavelength of 595 nm. The absorbance value is converted into a percentage of viable cells, and then the IC_{50} value is calculated using IBM SPSS Statistics version 25 (Probit/Logit).

Cell cycle analysis using a flow cytometer

Formulation with the lowest IC_{50} value (ZP 1:0) was used at concentrations of 75.0 and 150.0 µg/mL against WiDr (Liu et al., 2020). In a 12-well plate, cells were treated with ZP 1:0 extract and incubated at 37˚C for 24h. Then the cells were harvested and washed with 1X PBS, followed by adding 50 µg/ml PI solution and incubating for 30 min at room temperature in the dark. Subsequently, the cell cycle profile was determined using a flow cytometer (FACS Calibur) to quantify cell-cycle fractions using Cell Quest software (version 3.3; BD Biosciences).

Apoptosis cell analysis using a flow cytometer

The $5x10^4$ cells/well were incubated with extract for 24h, then harvested, washed, and resuspended with PBS and incubated for 15min at room temperature in 100µL 1X binding buffer containing 5µL Annexin V-FITC and 5µL PI. Then the apoptotic cells were identified using a flow cytometer (FACS Calibur) (Chen et al. 2018)

Observation of cell apoptosis using the double-staining method

The $5x10^4$ cells/well were directly attached to the coverslip placed in the 18-well plate, then washed using Phosphate Buffered Saline (PBS). The 1000µL of cell suspension was inserted into a microplate well, then covered with a slip and incubated in a 5%CO_2 incubator at 37°C for 24hours. Cells were treated with 1000 µL extract formulation, washed with PBS, stained with 10 µL acridine orange–ethidium bromide (100 µg:100 µg) for 3–5 min, and observed under a fluorescence microscope to distinguish viable (green) cells. (Liu et al. 2015).

Detection of caspase-8 and caspase-9 expressions using immunocytochemical methods

WiDr cells ($5×10^5$ cells/well) were cultured on coverslips in 24-well plates and incubated for

24 h to reach 80% confluence, then treated for 16 h at 37 °C with 5% CO_2. Cells were fixed with ice-cold methanol, blocked with hydrogen peroxide, incubated with caspase-8 or caspase-9 primary antibody followed by secondary antibody, and visualized with DAB. Counterstaining was performed with Mayer's hematoxylin, and cells were dehydrated in alcohol–xylol. Protein expression was observed under light microscopy, with brown/dark nuclei indicating positive expression, quantified as the percentage of positive cells (Susidarti et al., 2014)Brown cell staining indicates the presence of the target protein (Madjid & Hernowo, 2019).

Statistical analysis

To find the significant difference in values between extracts and test parameters, the data were statistically analyzed using the analysis of variance, followed by the Tukey test.

RESULTS AND DISCUSSION

Although ginger's chemopreventive potential is well studied, the anticancer activity of Z. officinale–P. reticulatus formulations remains unclear. We conducted initial in vitro cytotoxicity tests of three formulations against WiDr, MCF-7, and HeLa cancer cells.

In vitro cytotoxicity screening is a key preliminary method to evaluate the anticancer efficacy of plant extracts by assessing cell viability and calculating IC_{50} values. Z. officinale extract has been reported to show anticancer activity against various cancer cell lines, including cervical, prostate, breast, and colorectal cancer. (Saha et al., 2014) (Mao et al., 2019). Cytotoxicity was assessed using the MTT method to calculate IC_{50} values, where a lower IC_{50} indicates higher anticancer potency. Among the formulations, ZP 1:0 was more effective than ZP 0:1 and ZP 1:1, with the 1:1 combination eliminating ginger's inhibitory effect. Extracts with IC_{50} <100 ppm were selected for further anticancer mechanism studies.

MTT results showed that the extract combinations had no effect on MCF-7 cells (IC_{50}>1000µg/mL, not detected) and minimal effect on HeLa cells (IC_{50} 215–420µg/mL, not detected), whereas the ZP 1:0 formulation (ginger only) exhibited potential against WiDr cells (IC_{50} 102µg/mL) (Table 1). P. reticulatus showed no inhibitory effect on cancer cell growth, while combining it with Z. officinale (ZP 1:0) reduced ginger's activity against WiDr cells (IC_{50} 102 → 310 µg/ml). Therefore, the ZP 1:0 formulation (IC_{50} ~100 µg/ml) on WiDr cells was selected for further anticancer mechanism studies.

MTT assay showed that ZP 1:0 was the most potent formulation, while ZP 1:1 lost ginger's activity; extracts with IC_{50} <100 ppm were selected for further mechanism studies.

Staining of the cell

The WiDr cancer cells were subjected to two-fold labeling to quantify the percentage of viable versus deceased cells (Table 2 and Figure 2).

Table 1. The IC_{50} value of the ZP formulation against WiDr, HeLa, and MCF-7 cancer cells

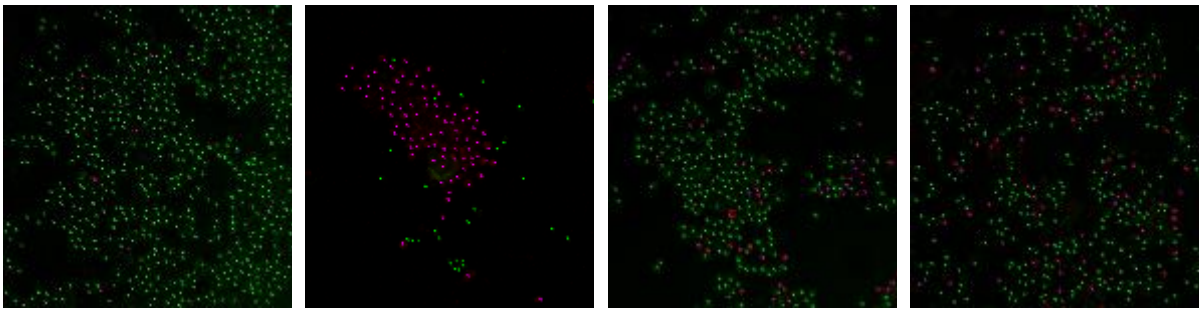
Extract formulation	IC_{50} value (µg/mL) of the extract against cancer cells		
	WiDr	HeLa	MCF-7
ZP 1:0	102.1 ± 23.7	214.7 ± 22.2	> 1000
ZP 0:1	ND	ND	> 1000
ZP 1:1	309.8 ± 16.4	415.9 ± 110.2	ND

ZP: *Z. officinale* and *P. reticulatus* ratio. ND: Cancer cells remain alive at all concentrations

Table 2. Effect of ZP 1:0 extract formulation treatment on the percentage of viable and dead WiDr cancer cells

Treatment	Concentration (µg/mL)	Percent of	
		Live cells	Dead cells
Cell control	-	99.54 ± 0.3^{d}	0.46 ± 0.3a
Doxorubicin	1.0	26.82 ± 12.8^{a}	73.18 ± 12.8^{d}
ZP 1:0	75.0	81.88 ± 1.4^{c}	18.01 ± 1.4^{b}
ZP 1:0	150.0	58.31 ± 4.3^{b}	41.69 ± 4.3^{c}

ZP: *Z. officinale-P* and *reticulatus* combination extract



(A) (B) (C) (D)

Figure 2. The staining of WiDr cancer cells by ZP 1:0 extract formulation treatment with *the AO-EB double staining* method. Control cell (A), Doxorubicin (B), ZP 1:0 75.0µg/mL (C), and ZP 1:0 150.0µg/mL

At a very small concentration (1.0µg/mL), doxorubicin killed almost all cancer cells (73.18%) while the 1:0 formulation of ZP extracts up to a concentration of 150.0 µg/mL kills only 41.69% of cells. The results suggest that the ZP 1:0 extract formulation caused dose-dependent death of WiDr cancer cells. At low concentrations (75µg/mL), mortality was 18.01% and increased to 41.69% (almost double) when the concentration was doubled (150µg/mL).

Anticancer mechanism of extract formulation ZP 1:0

The data demonstrated the effects of ZP 1:0 extract (75 and 150 µg/mL) on cell cycle distribution and caspase-8/9 expression in WiDr cells, compared with control and doxorubicin.

Cell cycle pattern

The cycle pattern of WiDr cancer cells by formulation treatment of ZP 1:0 extract was observed using a flow cytometer (Figure 3 and Table 3). The treatment of ZP 1:0 extract formulation at a lower concentration (75 µg/mL) showed a significantly increased percentage of cells in the G0-G1 phase (61.13%) compared to the untreated cell control (60.27%). The S phase (4.8%) decreased compared to the control without treatment (5.8%). At the higher concentration of *Z. officinale* extract (150 µg/ml), the G2-M phase percentage was significantly raised to 30.50%, compared to 15.00% in the control. At 150 µg/mL of the extract, the percentage of cells in the Sub-G1 phase increased to 5.80%, a significant rise from 0.23% in the control. The standard anticancer drug doxorubicin showed a lower percentage of cells in the G0-G1 phase (29.80%) and minimal Sub-G1 phase presence (0.23%).

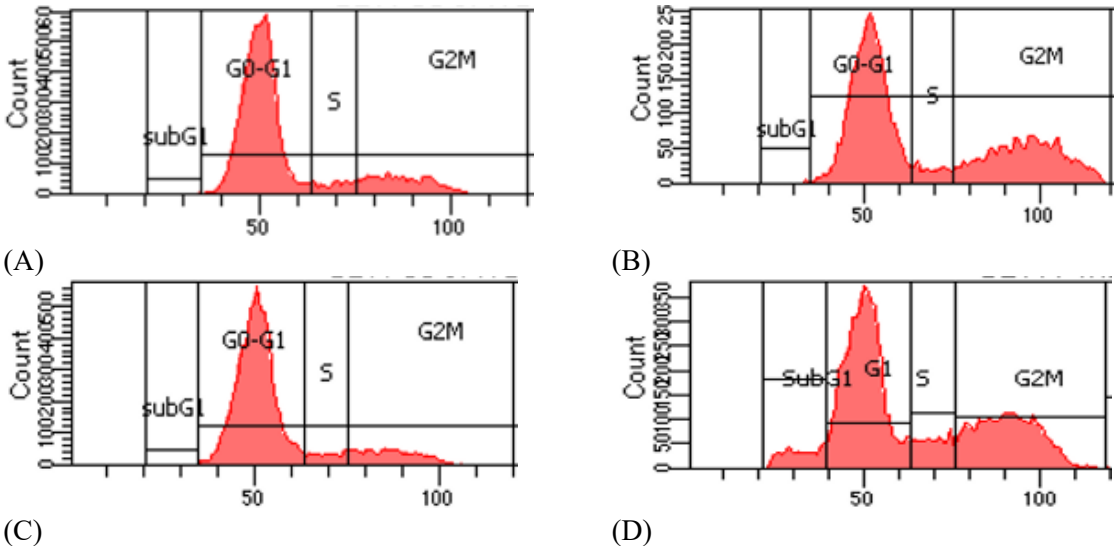


Figure 3. The cell cycle of WiDr cancer cell lines. Cell control (A), Doxorubicin treatment 1.0 µg/mL (B), Treatment of ZP 1:0 extract formulation at a concentration of 75 µg/mL (C), Treatment of ZP 1:0 extract formulation at a concentration of 150 µg/mL (D). ZP 1:0 at 75 µg/mL increased G0-G1 phase cells (61.13% vs. 60.27% control) and reduced S phase cells (4.8% vs. 5.8%). At 150 µg/mL, Z. officinale extract increased G2-M (30.50% vs. 15.00% control) and Sub-G1 (5.80% vs. 0.23% control), while doxorubicin, a standard anticancer drug, reduced G0-G1 (29.80%) with minimal Sub-G1 (0.23%).

Table 3. Phase distribution of WiDr cancer cell cycle by ZP 1:0 extract formulation treatment

Treatment	Concentration (µg/mL)	Percent (%)			
		Sub G1 (M)	G0-G1	S	G2-M
Cell control	-	0.23 ± 1.0^{a}	60.27 ± 1.4^{c}	$4.97 \pm 0,2^{b}$	15.00 ± 0.7^{b}
Doxorubicin	1.0	0.20 ± 0.0^{a}	29.80 ± 1.0^{a}	2.90 ± 0.1^{a}	19.07 ± 0.5^{c}
ZP 1:0	75.0	0.30 ± 0.0^{a}	61.13 ± 0.6^{c}	4.83 ± 0.3^{b}	12.53 ± 0.4^{a}
ZP 1:0	150.0	5.80 ± 0.6^{b}	45.07 ± 1.8^{b}	7.57 ± 0.5^{c}	30.50 ± 1.7^{d}

ZP: *Z. officinale* dan *P. reticulatus* extract combination. The different letters in the same column showed a significant difference in values between treatments.

In this study, the cytotoxic potential of ZP 1:0 extract formulation was dose-dependent. Both at low and high concentrations, the extract formulation has no significant effect on proliferation but induces apoptosis of WiDr cancer cells. Lima et al. (2018) stated that the results of their review, that ginger bioactive compounds, either from an extract or isolated bioactive compounds, showed significant activities, including anti-inflammatory, antiproliferative, anticancer, and antiinvasive. This corroborates previous findings of Abdullah et al. (2010) on the study of ethanolic extract of *Z. officinale* against 116 and HT 29 colon cancer cell lines. There is no antiproliferation effect of the ZP 1:0 extract formulation; it did not induce cell cycle arrest at the G0-G1 phase by an increased percentage of that phase compared to the untreated cell control. Proliferation inhibition in Abdullah et al. (2010) occurred at higher test concentrations than in this study, namely at 200 and 500 µg/mL of extract. This could be due to differences in test cells or the location of origin of the ginger samples. This condition is supported by the absence of a significant difference in S-phase values between the ZP 1:0 extract formulation at low concentrations and the control. According to Aras et al. (2014), phase S shows DNA replication activity. These results were corroborated by the double staining results, which showed that the 1:0 ZP extract formulation at low concentrations decreased the number of live cells compared to untreated cells, indicating the death of WiDr cancer cells.

Ginger extract has been reported to induce cell cycle arrest in G0-G1, though findings are fewer than those for G2-M arrest. Plant extracts were known for their capacity to induce G2-M phase cell cycle arrest in various cancer types and could be mediated through specific molecular pathways and the regulation of cell cycle proteins. In this study, the higher concentration of ZP 1:0 extract formulation (150 µg/mL) induced the G2-M arrest. This condition indicates that the extract at this concentration may prevent cells from completing mitosis, thereby halting cell division. It is also seen in the results of double staining, where there is cell death up to half the number of untreated cancer cells. Many researchers reported the induced G2-M phase cell cycle arrest effect by 6-gingerol from ginger on cancer cells through the mediation of specific proteins (Abdullah et al., 2010; Hu & Zhao, 2021; Luo et al., 2018; Mansingh et al., 2018). The arrest at G2-M is often associated with DNA damage that prevents progression to mitosis, leading to apoptosis in many cancer cells. These results were also clarified by the increase in the Sub-G1 phase, the phase that indicates the occurrence of apoptosis. It is suggested that the ZP 1:0 extract formulation at high concentration induced apoptosis. As a result of doxorubicin, there was a lower percentage of cells in the G0-G1 phase (29.8%) but no significant difference in the Sub-G1 phase.

Apoptosis distribution

In addition to cell cycle information, flow cytometers can also provide information about cell apoptosis. The assay using a flow cytometer presented the percentage of cell phases from live cells to dead cells (early apoptosis, late apoptosis, and necrosis) in the Q1–Q4 quadrant. Figures 4 and 5 present the distribution of WiDr cancer cell death type by doxorubicin treatment and ZP 1:0 extract formulation.

The control data showed the natural state of WiDr cells, with nearly all cells (96.73%) remaining viable and very few undergoing apoptosis or necrosis. Doxorubicin, a well-known chemotherapy drug, shows a significant reduction in live cells, with a marked increase in cells undergoing late apoptosis (51.97%) and necrosis (44.37%). This indicates doxorubicin's strong cytotoxic effect, as it drives a high proportion of cells toward apoptosis and necrosis, leading to cancer cell death.

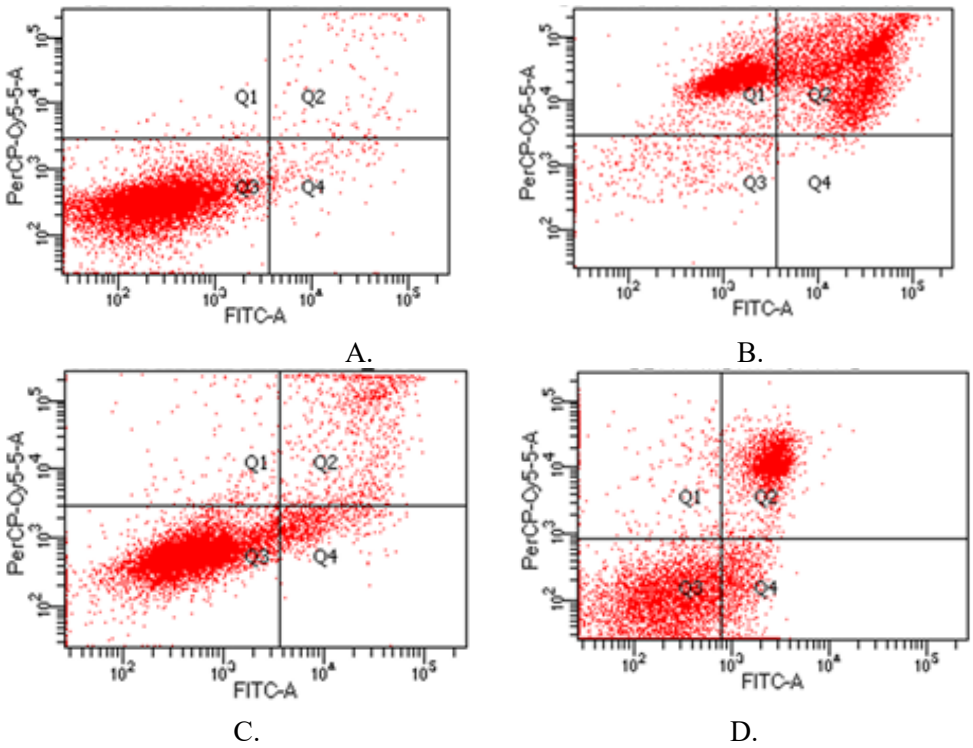


Figure 4. Distribution of apoptosis of WiDr cancer cells induced by ZP 1:0 extract formulation treatment 1:0 using a flow cytometer. Control cell (A), Doxorubicin (B), ZP 1:0 75.0 µg/mL (C), and ZP 1:0 150.0 µg/mL

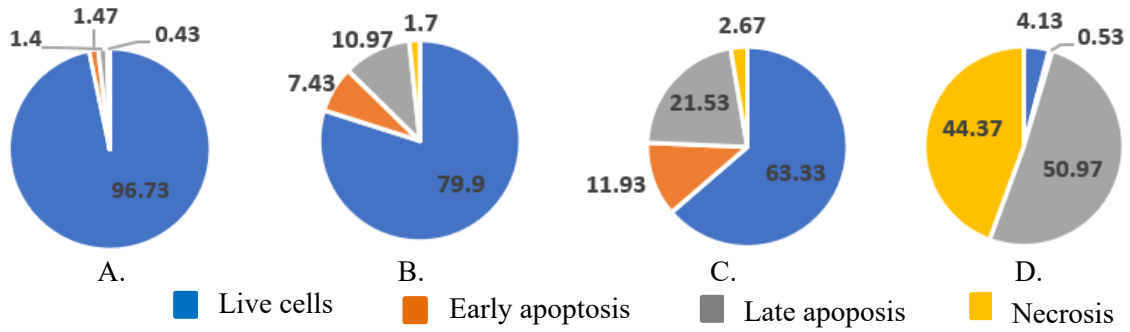


Figure 5. Distribution of apoptosis of WiDr cancer cells by ZP 1:0 extract formulation treatment using a flow cytometer. ZP: *Z. officinale-P* and *reticulatus* combination extract. Control cell (A), ZP 1:0 75.0 µg/mL (B), and ZP 1:0 150.0 µg/mL (C), Doxorubicin (D)

At 75 µg/ml, ZP 1:0 extract formulation resulted in a reduced percentage of live cells (79.90%) compared to the control (96.73%). There is an increase in early apoptosis (7.43%) and late apoptosis (11.93%), suggesting that this concentration initiates apoptosis in WiDr cells. The low necrosis percentage (1.70%) indicates a selective effect, as more cells are directed toward apoptosis rather than necrotic cell death. With an increased concentration (150 µg/mL), the live cell percentage drops further to 63.3%, with higher levels of early (11.93%) and late apoptosis (21.53%) than at 75 µg/mL. This concentration enhances the induction of apoptosis, which implies a dose-dependent response where higher extract concentrations increase apoptosis rates in WiDr cells. The relatively low necrosis (1.70%) compared to doxorubicin suggests that ZP 1:0 extract formulation may be less cytotoxic, favoring apoptosis over necrotic pathways. Numerous investigations have demonstrated that *Z. officinale's* ethanolic extract increases the induction of apoptosis in a dose-dependent way in a variety of cancer cell lines, including WiDr cells.

A study apoptosis of HeLa cells by ginger extracts showed a reduction in the number of cancer cells that are still alive, suggesting a shift toward necrosis and apoptosis (Nurani et al., 2024). The crude flavonoid extract of *Z. officinale* significantly induced apoptosis in HepG2 cells, evidenced by typical apoptotic changes (Elkady et al., 2017).

The types of apoptotic stages that occur in cells can be observed using a flow cytometer. The ZP 1:0 extract formulation at high concentrations showed increased cytotoxic effects compared to low concentrations in the early and late phases of apoptosis, and a small amount of necrosis. These results confirmed the study by Choi et al. (2022), that 6-gingerol-induced apoptosis in bladder cancer by activating caspase-3 and caspase-9 while Nedungadi et al. (2021) reported that ginger extract induced late apoptosis of MDA-MB-231 breast cancer through the translocation of apoptosis-inducing factor associated with DNA fragmentation. The standard cancer drug, doxorubicin, showed a much stronger effect with a predominance of advanced apoptosis and necrosis. This confirmed the effectiveness of doxorubicin as a potent anticancer agent but also indicated the possibility of high systemic toxic effects. The higher proportion of apoptosis compared to necrosis in the ZP 1:0 extract formulation indicates that the main mechanism of the ZP 1:0 extract formulation is most likely programmed apoptosis, which is more desirable in cancer therapy to reduce inflammation that often occurs with necrosis. Although ZP 1:0 extract formulation is less effective than doxorubicin, its more selective nature in inducing apoptosis with minimal necrosis could potentially reduce toxic side effects on normal cells in clinical applications. The induction of apoptosis without a significant amount of necrotic cell death implies that ginger extract may specifically target cancer cells while having less harmful effects on healthy cells—a crucial property that is desired in anticancer drugs. This difference suggests that while doxorubicin functions effectively, ZP 1:0 extract formulation may offer a complementary mechanism, particularly through G0-G1 and G2-M phase arrests.

These findings suggest that *Zingiber officinale* extract may be effective in inducing cell cycle arrest and apoptosis in cancer cells, supporting its potential as a complementary therapy in cancer treatment. The study by Sarmoko et al. (2020) demonstrated the potential of a complementary therapy combining 5-fluorouracil (5-FU) with red ginger extract, which increases cytotoxic activity against WiDr colon adenocarcinoma cells. While the study (Yusof et al., 2022) showed that by blocking the development of human colon cancer cells HCT 116 and HT 29 via caspase, the combination of Z. officinale and Piper betle extracts showed chemopreventive potential.

Analysis of cell death marker molecules

There are many marker proteins in the case of cancer, both activators and suppressors. In this study, we analyzed caspase-8 and caspase-9 as proteins that mark cell death (Table 5 and Figure 6).

Caspase-8 and caspase-9 are critical markers of apoptosis, where caspase-8 is typically associated with the extrinsic (death receptor) pathway and caspase-9 with the intrinsic (mitochondrial) pathway. Apoptosis signaling can occur through two different signaling pathways, namely *the death receptor pathway* (extrinsic pathway) and *the mitochondrial pathway* (intrinsic pathway). Caspase-8 is an executor protein in the extrinsic pathway. Extrinsic pathways are initiated through stimulation from *death receptors.* Caspase-9 is an initiator of apoptosis on the intrinsic pathway. Intrinsic pathways are initiated through the release of signaling factors from mitochondria.

Table 5. Effect of ZP 1:0 extract formulation treatment on the caspase-8 and caspase-9 expression of WiDr cancer cell lines

Treatment	Concentration (µg/mL)	Expression (%) of	
		Caspase-8	Caspase-9
Cell control	-	52.35 ± 3.7^{a}	13.64 ± 3.1^{a}
Doxorubicin	1.0	67.12 ± 6.2^{b}	47.95 ± 6.9^{b}
ZP 1:0	150.0	96.82 ± 6.4^{c}	89.67 ± 9.4^{c}

ZP: *Z. officinale-P* and *reticulatus* combination extract

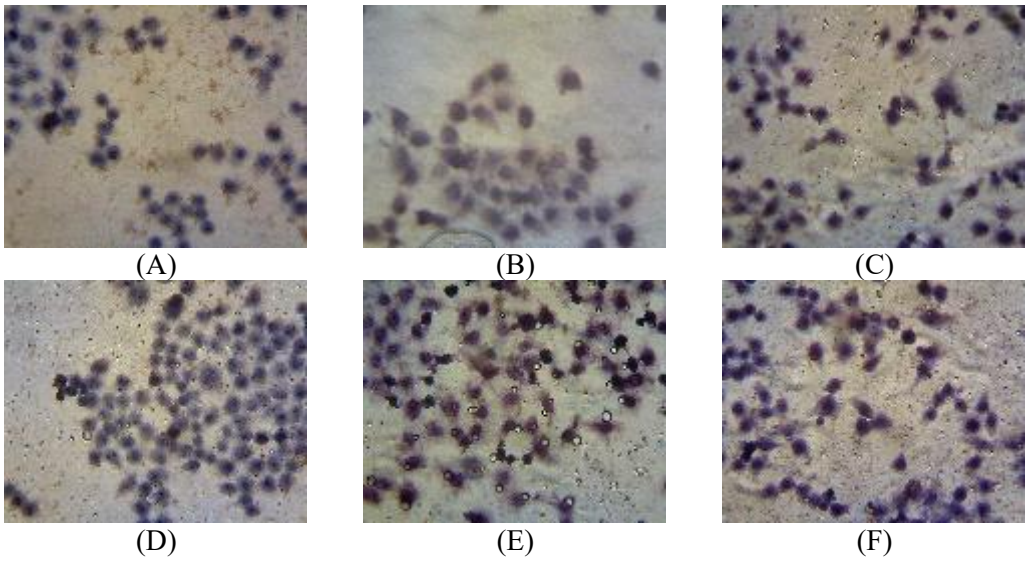


Figure 6. Expression of caspase-8 (A-C) and caspase-9 (D-F) in WiDr cells by ZP 1:0 extract formulation treatment using the immunocytochemical method. Cell control (A), Doxorubicin treatment 1.0 µg/mL (B), ZP 1:0 extract formulation treatment 75 µg/mL (C)

Doxorubicin elevated both caspase-8 and caspase-9, significantly increasing caspase-9 levels, which suggests it activates apoptosis primarily through the intrinsic pathway while also involving the extrinsic pathway to a lesser degree. Treatment with ZP 1:0 extract formulation caused a substantial increase in both caspase-8 and caspase-9 expression. At 75 µg/ml, there's a strong activation of caspase-8, which points to the activation of the extrinsic apoptosis pathway. The combined extract of *Z. officinale* and *Piper beetle* significantly increased caspase-8 and caspase-3 expression in both HCT116 and HT29 cells Yusof et al., 2022), but this study did not report on the expression of caspase-9. The higher concentration (150 µg/mL) shows increased caspase-9 expression, indicating enhanced activity of the intrinsic pathway as well. This dose-dependent response suggests that ZP 1:0 extract formulation can activate multiple apoptotic pathways (both of the intrinsic dan extrinsic pathways) in WiDr cancer cells, contributing to its anticancer efficacy.

This study presents several new findings compared to previous research. Extracts of P. reticulatus, whether alone or in combination with Z. officinale, exhibit strong antioxidant properties (Kristiani et al., 2024), but do not demonstrate cytotoxic activity against cancer cells. Z. officinale extract exhibits strong cytotoxic activity against WiDr cancer cells, while its activity against HeLa and MCF-7 cancer cells is weak. Furthermore, it has been verified that Z. officinale extract inhibits the growth of WiDr cancer cells through the induction of apoptosis via both intrinsic and extrinsic pathways. The anticancer mechanism of Z. officinale extract is selective for apoptosis, which offers the advantage of minimizing damage to normal cells.

This finding has a significant scientific and societal contribution from the scientific validation for the use of *Z. officinale–P. reticulatus* formulations as an alternative cancer therapy. The findings could improve public health outcomes, promote the sustainable use of local medicinal plants, and help develop affordable, natural anticancer medications for society, particularly in underdeveloped areas

CONCLUSION

The ZP 1:0 ethanolic extract of *Zingiber officinale* demonstrated notable anticancer activity against WiDr colon cancer cells (IC_{50} 102.1 µg/mL), inducing both early and late apoptosis via caspase-8 and caspase-9 pathways with minimal necrosis. Its selective pro-apoptotic mechanism suggests potential as a safer, plant-based adjunct for colon cancer therapy, though less potent than doxorubicin. Further identification and fractionation of active compounds are recommended to enhance efficacy. This study underscores the scientific and social value of developing regional medicinal plants like *Z. officinale–P. reticulatus* as affordable natural anticancer agents, especially for resource-limited settings.

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AUTHOR CONTRIBUTION STATEMENT

All authors contributed significantly to the work reported in this manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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