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Original Research Article

Cytotoxic and Antiproliferative Effects of Yellow Passion Fruit (*Passiflora edulis* f. flavicarpa) Juice Against T47D Breast Cancer and HeLa Cervical Cancer Cell Lines

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ABSTRACT

Several reports have shown the antitumor activity of yellow passion fruit (YPF) (*Passiflora edulis* f. flavicarpa), but limited studies are exploring its mechanism. This study aimed to examine the antitumor mechanism of YPF juice on T47D and HeLa cell lines. The YPF juice was administered at doses of 0.25 IC_{50}, 0.5 IC_{50}, and IC_{50}. The cytotoxic mechanism of YPF juice was determined by assessing the antiproliferative effects and apoptosis induction. The antiproliferative effects were assessed based on doubling time with MTT assay, while apoptosis induction and cell cycle were examined using flow cytometry with annexin-V and propidium iodide staining. The results showed that the doubling time of T47D and HeLa cells treated with YPF juice was longer than control cells. There was an increase significantly in the average number of apoptosis of T47D and HeLa cells treated with YPF juice. At a dose equivalent to IC_{50}, the HeLa cell cycle was inhibited, leading to a 13% reduction in the G0-G1 phase. Whereas in T47D cells, almost all cells were in the sub-G0 phase, indicating cell cycle arrest across all phases. The results proved that YPF juice inhibited the cell cycle and increased apoptosis of T47D breast cancer cells and HeLa cervical cancer cells.

Keywords: yellow passion fruit, *Passiflora edulis* f. flavicarpa, T47D, HeLa, flow cytometry

Introduction

Many plants found worldwide, including *Jatropha curcas*, *Pyrenacantha staudtii*, *Picralima nitida*, *Jatropha gossypifolia*, and *Passiflora edulis* f. flavicarpa (Yellow passion fruit), possess anticancer potential. The first four plants, originating from Africa, have demonstrated strong anticancer properties with IC_{50} values ranging from 23-38 µg/ml.[1] Meanwhile, Yellow passion fruit (YPF) is rich in essential nutrients such as vitamin A, carotenoids, and polyphenols, known for their antioxidant properties.[2,3] Several studies also reported the anticancer properties of nutrients derived from YPF extract. In addition, the plant was shown to possess a high total carotenoids and polyphenols content of 9.25 mg/L[4] and 435 mg/L[5], respectively. These carotenoids include lycopene, *α*-carotene, *β*-carotene, and *β*-cryptoxanthin.[6] Previous research has demonstrated the role of antioxidant compounds from plants in exerting anticancer effects.[7]
The active content of YPF can also inhibit the growth of leukemia cancer cells and induce apoptosis at different phases. According to previous studies, inhibition of the G0/G1 phase of the cell cycle and apoptosis induction in HL-60 myeloid leukemia cells could be attributed to its carotenoid compounds.[8]

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In the context of herbal plants, YPF has been extensively used in traditional medicine for the treatment of various diseases, including intestinal antitumors.[9] Various studies also proved the inhibitory effect of its MMP-2 and MMP-9 proteins, which play an essential role in tumor invasion, metastasis, and angiogenesis.[10] In addition, YPF has antioxidant activity and can reduce the viability of colon cancer cell lines.[11] These results necessitate further exploration of inherent bioactive compounds and their mechanisms in preventing and controlling carcinogenesis, particularly in T47D breast cancer and HeLa cervical cancer cell lines.

Materials and Methods

This research has received approval from the Ethics Committee of the Faculty of Medicine, Universitas Islam Sultan Agung, through certificate number 306/IX/2022/Komisi Bioetik.

Plant material and Yellow passion fruit (YPF) preparation
Mature YPF were collected locally in March 2023 and authenticated by Integrated Biomedical Laboratory Universitas Islam Sultan Agung Semarang, with specimen number FKSA-PE1-III23. The collected fruits were cut in half, and the flesh and seeds were taken. The samples were then weighed to 1000 grams, and placed in a juicer to obtain YPF juice. Subsequently, the juice was divided into 7 concentrations, including 0.39%, 0.78%, 1.56%, 3.12%, 6.25%, 12.5%, and 25%. The process was then continued with dilutions using the formula M1 × V1 = M2 × V2.

Determining the Dosage of YPF Juice
The IC_{50} value of YPF juice was determined by testing the cytotoxic effect on the T47D breast cancer cell line, obtaining a value of 12.07 µg/ml. Furthermore, the dosage of YPF juice was determined in 3 concentration ranges, namely 0.25 IC_{50} of 3.02 µg/mL (dose 1), 0.5 IC_{50} of 6.03 µg/mL (dose 2), and IC_{50} of 12.07 µg/mL (dose 3). As for the HeLa cervical cancer cell line, the IC_{50} value of YPF juice was 10.40

µg/ml. Furthermore, the dosage of YPF juice was determined in 3 concentration ranges of 2.6 µg/mL, 5.2 µg/mL, and 10.40 µg/mL.

Cytotoxic Test on T47D Breast Cancer and HeLa Cervical Cancer Cell Lines

Cytotoxic tests were performed using Roswell Park Memorial Institute (RPMI)-1640 medium. A total of 100 µl T47D breast cancer and HeLa cervical cancer cell line suspension with a density of 1 × 104 cells/100 µl was distributed into a 96-well plate and incubated for 24 hours. Subsequently, 100 µl of test solutions with various series of concentrations were added to the well. As a media control, 100 µl doxorubicin in various concentration series was added to a well containing 100 µl of culture medium and 100 µl of cell suspension. For the control cell, 100 µl of culture medium was added to a well containing 100 µl of cell suspension. All wells were then incubated for 24 hours in the incubator with a flow of 5% CO_2 and 95% O_2.

At the end of the incubation, the culture media was removed, followed by the addition of 10 µl of MTT solution (5mg/ml PBS). The medium was then replaced with 190 µL RPMI-1640 medium and cells were incubated for 3-4 hours. MTT reaction was stopped by the addition of 100 µl SDS stopper reagents. Furthermore, the well plate was then wrapped in tissue and incubated in a dark room for one night at room temperature. Living cells reacted with MTT, leading to the formation of a purple coloration. The test results were read with Elisa Reader (BioTek) at a wavelength of 595 nm.[12]

Apoptosis Test and Cell Cycle Phase Detection

After confirming that cells were prepared for treatment, the media in each well was removed using a pipette. The wells were then washed with 1 mL of PBS solution per well. Subsequently, the prepared test substance solution was added to each well at a volume of 2 mL per well, following the labels on the well plate. The 3 plates were reincubated for 24 hours in a CO_2 incubator (Thermo Fisher Scientific) at a temperature of 37°C.

The well plate, previously incubated with samples, was retrieved and transferred to a labeled conical tube using a 1 mL micropipette, followed by the addition of 500 µL PBS solution. A 200 µL solution of 0.25% trypsin-EDTA was added and incubated for 3 minutes at 37ºC. A 1 mL Culture medium was added to inactivate trypsin, and the mixture was resuspended until cells detached individually, followed by observation under an inverted microscope. After individual detachment, cells were transferred to a conical tube, and an additional 500 µL PBS solution was added to the well to collect any remaining cells, which were transferred to the conical tube. The conical tube was then centrifuged at 600 rpm for 5 minutes, and the resulting supernatant was discarded. The cell pellet was washed with 500 µL cold PBS solution, and it was centrifuged again. Subsequently, the supernatant was discarded, followed by the dropwise addition of 500 µl of 70% alcohol (1 drop per second) into the conical tube while gently shaking. The conical tube was then stored at room temperature (37ºC) for 30 minutes and the alcohol was then discarded. A total of 500 µL PBS solution was added, followed by centrifugation at 2000 rpm for 3 minutes, and washing with PBS solution was repeated twice. The conical tube was wrapped in aluminum foil and labeled, and 30 µL of Annexin V reagent and 50 µg/mL Propidium iodide (50 times) were added to 500 µL PBS solution. Cells were then incubated in the dark for 10-15 minutes at a temperature of 15-25°C. Cell suspension was transferred to a flow cytometry tube and analyzed using flow cytometry (Beckman Coulter) to determine the number of cells undergoing apoptosis.

Data Analysis

The data collected were subjected to descriptive analysis, normality testing using the Shapiro-Wilk test, and homogeneity testing using the Levene Test. When the data exhibited normal distribution and homogeneity, the hypotheses were tested using One-Way ANOVA, followed by a post hoc test.

Results and Discussion

The Observational Results of YPF Juice Effects on HeLa Cell Cycle

The results of observing the effect of YPF juice on the cell cycle of HeLa cells using flow cytometry are presented in Figure 1. The figure revealed differences in cell cycle profiles between control HeLa cells and those treated with YPF juice or Doxorubicin. Furthermore, HeLa cells were used in this study as a model representing the characteristics of highly proliferative cells, which was one of the traits of cancer cells. A previous study revealed that proliferative capacity refers to the ability of cells to grow and develop.[12] The inhibition of the proliferation of cancer cells often causes a cessation in division, leading to death.

From the results of flow cytometry, the average percentage of HeLa cells in each cell division phase was calculated, as shown in Table 1. The results showed that the administration of YPF juice exhibited regulatory activity on the cell cycle of cervical cancer HeLa cells. The administration of YPF juice at 0.5 IC_{50} dose led to an increase in the percentage of cells in the G0-G1 phase (72.9%) compared to the control (59.7%). Furthermore, there was a decrease in the number of cells in the S and G2-M phases, with respective increments of 14% and 13.9% compared to the control cell, which showed percentages of 17.5% in the S phase and 23.6% in the G2-M phase.

Based on the results, treatment with YPF juice at an IC_{50} dose led to a 13% inhibition of the cell cycle in the G0-G1 phase. The decrease in the number of samples in the G0-G1 and S phases indicated cell cycle inhibition, while the increase in the M1 phase suggested apoptosis, preventing cells from progressing in the cycle for division. Carotenoids, present in YPF juice in this study, could function to inhibit cell proliferation and reduce the risk of cancer.[13]

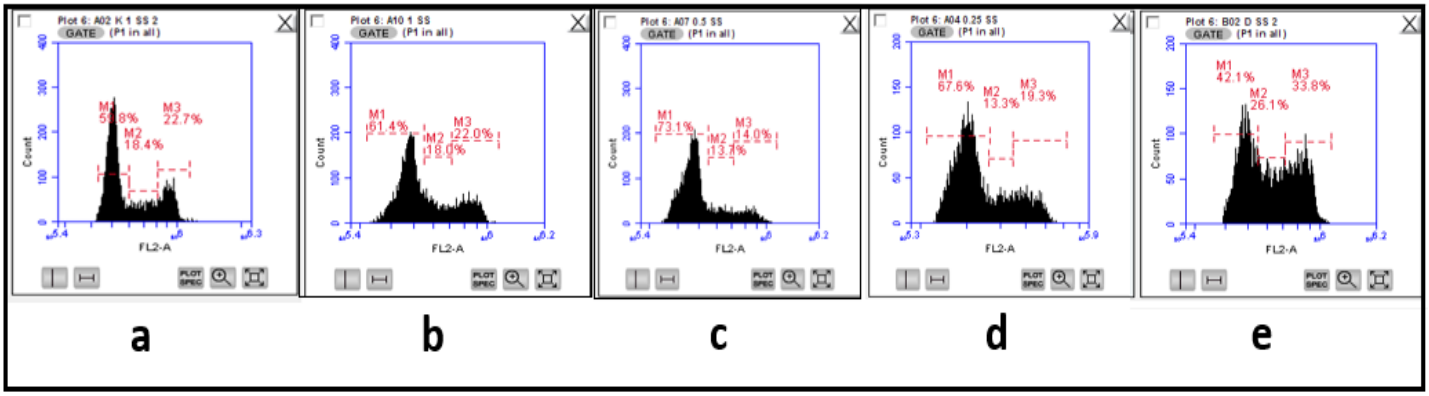

Figure 1: Flow cytometry representation of HeLa cell cycle: (a) control HeLa cells, (b) HeLa cells treated with YPF juice at IC_{50} dose, (c) 0.5 IC_{50}, (d) 0.25 IC_{50}, and (e) HeLa cells treated with Doxorubicin.

The Observational Results of YPF Juice Effects on T47D Breast Cancer Cell Cycle

The results of observing the impact of YPF juice on the T47D breast cancer cell cycle using flow cytometry were presented in Figures 2 and 3. In Figure 2, different doses of YPF juice led to distinct T47D breast cancer cell cycle profiles. Meanwhile, Figure 3 illustrates differences in cell cycle profiles between control T47D breast cancer cells and those treated with YPF juice or Doxorubicin. The analysis results indicated that treatment with YPF juice had a positive effect in inhibiting T47D breast cancer cell proliferation. This was indicated by a decrease in the percentage of cells undergoing proliferation compared to the control. In addition, these results were consistent with previous reports, suggesting that flavonoids were also present in YPF, and acted as potent antioxidants under normal conditions and pro-oxidants under pathological conditions. This dual role activated apoptosis, suppressed proliferation, and reduced inflammation.[14]

Figure 2 showed that the differences in YPF juice doses modified the T47D breast cancer cell cycle. With the increased doses, T47D breast cancer cells entering the division cycle decreased. At the dose corresponding to IC_{50}, almost all cells were in the sub-G0 phase, indicating cell death.

From the results of flow cytometry, the average percentage of T47D cells in each cell division phase was calculated, as shown in Table 2. In addition, treatment with YPF juice at IC_{50} dose led to cell cycle inhibition, across all phases. The results suggested that YPF juice induced apoptosis by inhibiting the more active cancer cell line in T47D.

Apoptosis was regulated by cell cycle-regulator genes, such as the p53 gene expressed by the p53 protein. When DNA damage occurred, p53 often underwent overexpression to initiate DNA damage repair. DNA repair typically occurred before the S phase through the cell cycle arrested at the G1 phase until recovery was complete.[15]

Mechanism of Cytotoxicity Against T47D Breast Cancer and HeLa Cervical Cancer Cell Lines

Cytotoxic and antiproliferative activities were evaluated to determine whether the effect of YPF juice correlated directly with inducing cell death or suppressing cell proliferation.[16] A graph depicting the relationship between viable cells and incubation time for T47D and HeLa cells is shown in Figure 4.

The calculated number of viable cells at each incubation time was used to determine cell proliferation inhibition ability by calculating doubling time. Furthermore, doubling time was calculated using the regression equation between incubation time and the number of cells. In this study, the value of this parameter could be obtained from the slope of the equation. The larger the slope value, the shorter the doubling time, and vice versa. The results of the linear regression equation and doubling time calculations for each dose are presented in Table 3 and Table 4.

Based on the results, YPF juice had growth-inhibitory activity on cancer cells. This was observed in the doubling time of T47D breast cancer and HeLa cervical cancer cells, where the administration showed a greater impact compared to the control. The growth profile of cancer cells under YPF juice treatment experienced inhibition, as shown in Table 3 and Table 4. Therefore, it demonstrated the ability to inhibit the proliferation of T47D breast cancer and HeLa cervical cancer cells, as well as cytotoxic activity. In this study, cytotoxic activity was expressed in IC_{50} value, while antiproliferative effects were reflected in doubling time value.[17]

Table 1: The average percentage of HeLa cells in each cell division phase

Group	G0-G1 Phase (%)	S Phase (%)	G2-M Phase (%)
Control cell	59.7	17.5	23.6
YPFJ 2.6 µg/ml	67.8	13.2	19.3
YPFJ 5.2 µg/ml	72.9	14.0	13.9
YPFJ 10.40 µg/ml	62.5	17.6	21.1
Doxorubicin	42.1	26.2	34.8

YPFJ: Yellow passion fruit juice

Table 2: Average percentage of T47D breast cancer cells in each cell division phase

Group	G0-G1 Phase (%)	S Phase (%)	G2-M Phase (%)
Control cell	53.2	19.4	25.8
YPFJ 3.02 µg/ml	54.0	17	28.8
YPFJ 6.03 µg/ml	50.3	23.4	22.9
YPFJ 12.07 µg/ml	24.8	47.4	19.8
Doxorubicin	56.7	10.1	33.1

YPFJ: Yellow passion fruit juice

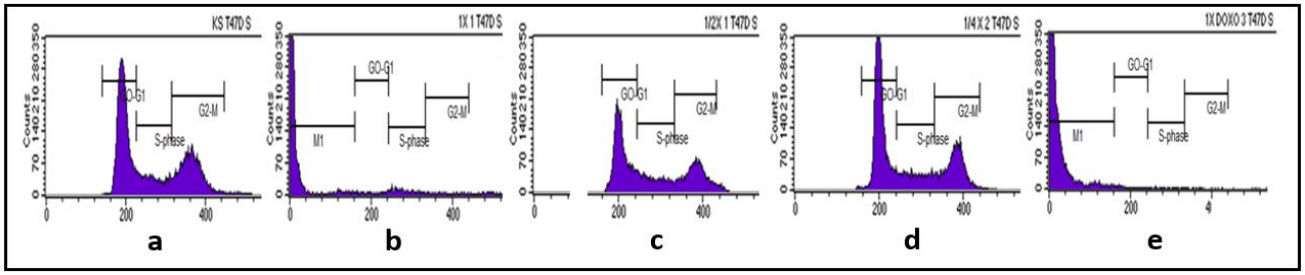

Figure 2: Flow cytometry representation of T47D cell cycle: (a) control T47D cells, (b) T47D cells treated with YPF juice at IC_{50} dose, (c) 0.5 IC_{50}, (d) 0.25 IC_{50} and (e) T47D cells treated with Doxorubicin.

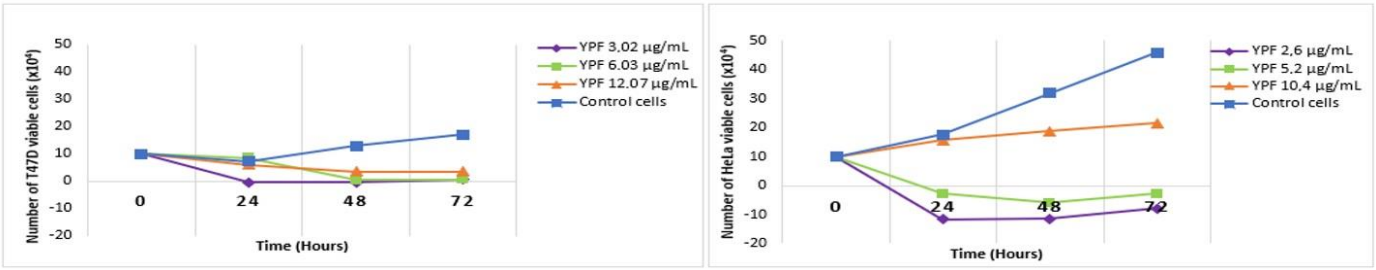

Figure 3: Correlation between the number of T47D and HeLa viable cells versus incubation time in treatment and control groups.

The doses of YPF juice given to the samples were 3.02 µg/ml, 6.03 µg/ml, and 12.07 µg/ml, respectively, which represented 0.25 IC_{50}, 0.5 IC_{50}, and IC_{50}. All viable cells, as well as cells undergoing early apoptosis, late apoptosis, and necrosis, were quantified using a flow cytometer.

The flow cytometry method was selected because Annexin V and Propidium Iodide staining could be used to identify cells undergoing early apoptosis, late apoptosis, and necrosis. Furthermore, apoptosis testing used Annexin V conjugated with fluorescein isothiocyanate (FITC) to bind phosphatidylserine (PS) on the cell surface. According to previous studies, phosphatidylserine (PS) was an aminophospholipid that translocated from the inner to the outer membrane during apoptosis. Annexin V was a protein with a high affinity for negatively charged phospholipids in the presence of Ca^{2+} ions. The calcium in Annexin V was bound to phosphatidylserine (PS) on the outer plasma membrane and underwent apoptosis.[18]

The results of flow cytometry for T47D breast cancer cell lines are presented in Figure 4 and for HeLa cervical cancer cell lines are presented in Figure 5. The results in the control cell group showed a viability percentage of 94.27% and a necrosis percentage of 0.57%. The viability percentage in the control cell group was higher compared to others. The calculated results of the average early apoptosis and late apoptosis using a flow cytometer were shown in the bar chart of Figures 4 and 5.

In Figure 6, the results showed that the highest average apoptosis occurred in T47D cells and HeLa cells that received the highest dose of YPF juice (a dose equivalent to IC_{50}). The apoptosis induction effect occurs in a dose-dependent manner. The increase in the incidence of apoptosis and necrosis in the samples was due to critical DNA damage and poor repair capacity caused by increased DNA oxidation from active compounds in YPF juice.[10]

The normality test using the Shapiro-Wilk method gave a p-value > 0.05, indicating that the data were normally distributed. However, the homogeneity test with Levene's test yielded a p-value < 0.05 (0.003), indicating non-homogeneous variance in the data. Based on these results, the data could be tested with One-Way ANOVA, followed by the Post-Hoc Tamhane statistical test.

The statistical test using One-Way ANOVA showed a significance value of 0.000 ($p < 0.05$), showing that YPF juice had a significant effect on the average apoptosis. The statistical test was further followed by the Tamhane post-hoc test with the results presented in Table 5.

Post-hoc test results indicated significant differences ($P < 0.05$) between the control group and samples receiving YPF juice doses of 6.04 µg/ml, 12.07 µg/ml, and doxorubicin. Significant differences were also observed between the group receiving a dose of 3.02 µg/ml and those administered with 12.07 µg/ml and doxorubicin. In addition, the group receiving a YPF juice dose of 6.03 µg/ml showed significant variations from the control group, as well as samples administered with 12.07 µg/ml and doxorubicin. The group receiving a dose of 12.07 µg/ml exhibited significant differences from the controls, as well as those administered with 3.02 µg/ml and 6.03 µg/ml. Furthermore, the doxorubicin group showed significant differences from the controls, and samples were given 3.02 µg/ml and 6.03 µg/ml.

Results indicating non-significant differences were found between the controls and the group receiving a YPF juice dose of 3.02 µg/ml. The group administered a dose of 3.02 µg/ml showed non-significant differences with the control and samples treated with a dose of 6.03 µg/ml. In addition, non-significant variations were recorded between the groups receiving doses of 6.03 µg/ml and 3.02 µg/ml. Samples administered with a dose of 12.07 µg/ml showed non-significant differences with the doxorubicin group, which also differed from those treated with a dose of 12.07 µg/ml. The highest average apoptosis in the group that received YPF juice with a dose of 12.07 µg/ml, reached 89% and confirmed that the cell population in the sub-G0/G1 phase was associated with cell death mechanisms (apoptosis or necrosis). Factors influencing cell cycle progression by disrupting protein signals were linked to various cellular processes, including cell proliferation, apoptosis, cell migration, and DNA repair.[19,20] The increase in apoptosis levels could be associated with the presence of flavonoids, quinones, sterols, and glycosides found in YPF juice.[21]

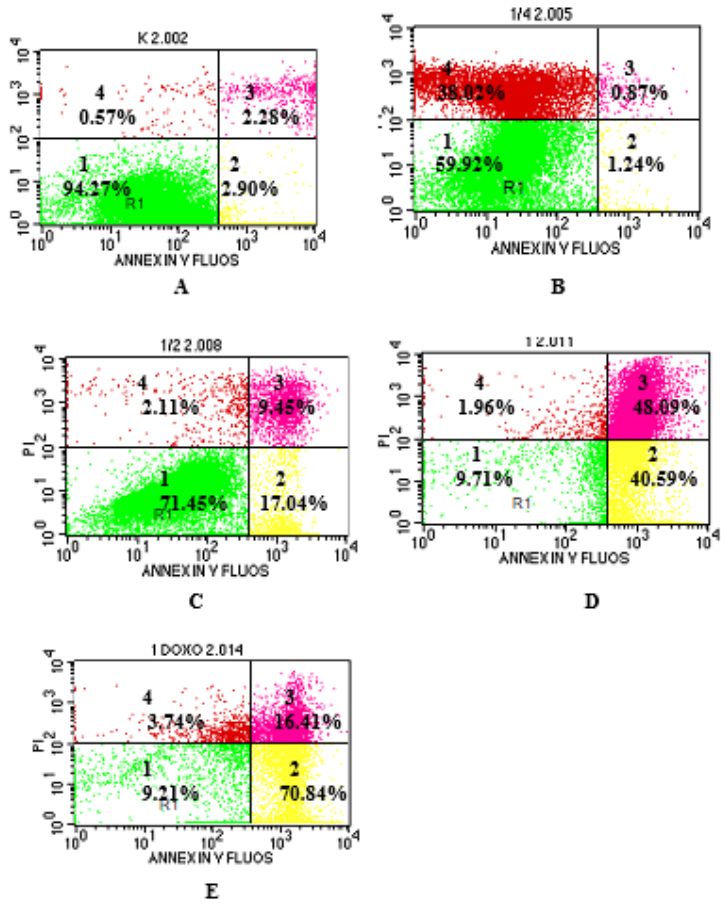

Figure 4: Flow cytometry outcomes for T47D breast cancer cell line.

Key: 1. Viable cells; 2. Early apoptosis; 3. Late apoptosis; 4. Necrosis (A) Control Cell; (B) YPF juice dose 3.02 µg/ml; (C) YPF juice dose 6.03 µg/ml; (D) YPF juice dose 12.07 µg/ml; (E) Doxorubicin dose.

Table 3: Linear regression equations and doubling time values for T47D breast cancer under various treatments of YPF juice concentrations

Concentration	Equation	Slope	Doubling Time (Hours)
12.07 µg/mL	y = 5404.8x+21719	5404.8	3.6 hours
6.03 µg/mL	y = -8846.6x+48986	-8846.6	3.27 hours
3.02 µg/mL	y = -13983x+69947	-13983	-454 hours
Cell Control	y = -36.28x+3512.5	53888	1.1 hours

Table 4: Linear regression equations and doubling time values for HeLa cervical cancer cells under various treatments of YPF juice concentrations.

Concentration	Equation	Slope	Doubling Time (Hours)
10.40 µg/ml	y = 3758.6x + 7050.8	3758.6	3.44
5.2 µg/ml	y = -4186x + 10008	-4186	-2.38
2.6 µg/ml	y = -5355.1x + 8147.3	-5355.1	-2.21
Cell Control	y = 12145x – 4050.2	12145	1.31

Table 5: Average apoptosis difference test of T47D breast cancer cell between two groups with Tamhane Post-Hoc test.

	Control cell	3,02 µg/ml	6,04 µg/ml	12,07 µg/ml	Doxorubicin
Control cell	-	1.000	0.000*	0.000*	0.000*
YPFJ 3.02 µg/ml	1.000	-	0.089	0.005*	0.005*
YPFJ 6.03 µg/ml	0.000*	0.089	-	0.000*	0.000*
YPFJ 12.07 µg/ml	0.000*	0.005*	0.000*	-	0.128
Doxorubicin	0.000*	0.005*	0.000*	0.128	-

YPFJ: Yellow passion fruit juice

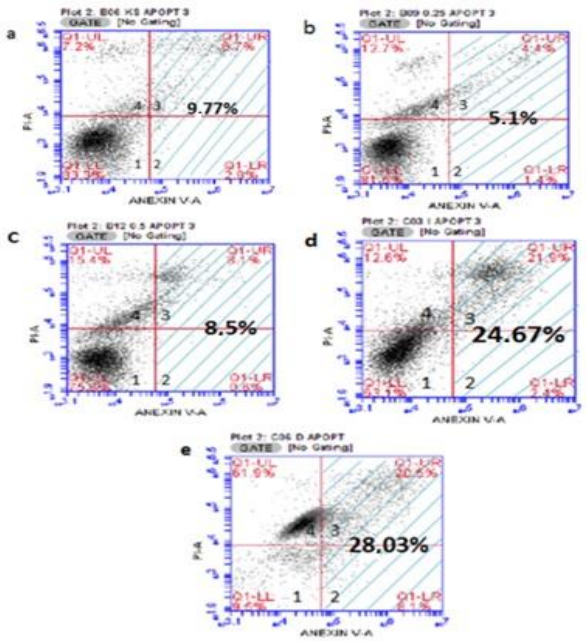

Figure 5: Flow cytometry results of HeLa cervical cancer cells. a. Cell Control; b. YPF dose 2.6 µg/ml; c. YPF dose 5.2 µg/ml; d. YPF dose 10.4 µg/ml; e. Doxorubicin. In each graph, quadrants 1, 2, 3, and 4 respectively show life cells, early apoptosis, late apoptosis, and necrosis. The diaries area in each graph is the percentage of total cells that experience apoptosis.

Conclusion

In conclusion, the results showed that YPF juice affected the cell cycle of T47D breast cancer and HeLa cervical cancer cells, and had a cytotoxic mechanism by inhibiting proliferation and increasing apoptosis. IC_{50} dose induced cell cycle inhibition in HeLa cervical cancer cells, leading to a 13% reduction in the G0-G1 phase. Whereas in T47D breast cancer cells, YPF juice decreases the cell cycle. At the dose corresponding to IC_{50}, almost all cells were in the sub-G0 phase, indicating cell cycle arrest across all phases.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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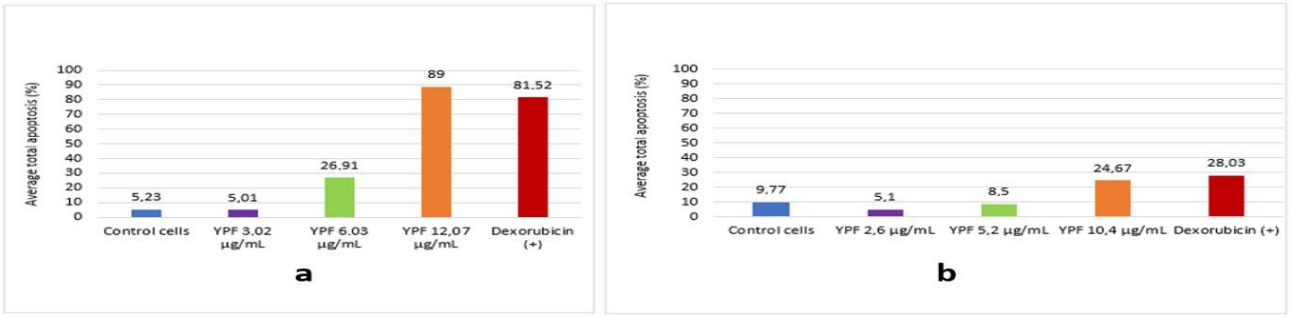

Figure 6: Average total apoptosis, (a) T47D breast cancer cells, and (b) HeLa cervical cancer cells in each group.

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