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Enhancing the effectiveness of paclitaxel with stigmasterol bioactive compounds for cancer treatment

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Abstract

This study examines the anticancer activity of stigmasterol bioactive compounds combined with paclitaxel. Drug resistance and high toxicity in chemotherapy present a challenge in treating cancer. However, no study has reported the combination therapy of SS with PTX up to now. We evaluated the potential effects of stigmasterol (SS) combined with Paclitaxel (PTX) on cell viability and apoptosis in liver cancer (HepG2) and normal liver (AML 12) cells. The SS showed the highest cytotoxic effects on HepG2 cells (IC_{50}= 117.75 µg/ml) compared to PTX (IC_{50}= 294.59 µg/ml); however, SS and PTX did not affect normal cells, and SS caused higher apoptotic activity in the sub-G1 phase in HepG2 cells after being treated with SS alone compared to other treatments. In combination therapy for both cells, PTX: SS significantly increased cell viability (IC_{50} of 464.44 and 1056.44 µg/ml for HepG2 and AML 12, respectively) that differed from the drug alone and the compound, confirming that the PTX combined with SS did not enhance cytotoxic performance against HepG2 cell lines. However, this study found that PTX: SS could reduce the side effects arising from the treatment with PTX. This research indicates that SS is a potential anticancer agent for liver cancer. Furthermore, the current study introduces a novel synergistic therapy that can target various cell lines or enhance the concentration of both SS and PTX to investigate the delivery technologies of both compounds.

Keywords: Apoptosis, Combination therapy, Drug resistance, HepG2 cells, Paclitaxel, Stigmasterol.

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1. Introduction

Because liver cancer (HepG2) is widespread in Asia, Europe, and the United States [1] a need exists to find new adjuvants or drugs to overcome toxicity and drug resistance [2]. Plant sterols are specific compounds with anti-cancer, anti-diabetic, and anti-inflammatory pharmacological properties. Many vegetables and foods contain Stigmasterol (SS, $C_{29}H_{48}O$), the most common sterol [3]. Recently, SS has attracted more attention due to its anti-cancer activities against different cancer cell lines through severalmechanisms such as suppression of cancer cell growth, tumor angiogenesis inhibition, growth of cholangiocarcinoma, and apoptosis induction, which may be due to the interactions of mRNA and double bond cell protein of SS at the C-5 and C-22 positions [3, 4].

Paclitaxel (PTX) has shown effectiveness against several cancer cells, either *in-vitro* or *in-vivo*, or clinical applications for melanoma, ovarian cancer, non-small cell lung cancer, metastatic breast cancer, and other types of cancer [5]. PTX causes cell death by binding to the microtubules of cells and/or arresting the cell cycle at the G2/M phase [6]. The PTX formula consists of ethanol and Cremophor EL, which can cause several side effects, such as peripheral neuropathy and hypersensitivity reactions. Therefore, developing an aqueous formula for PTX is essential [7]. However, based on multidrug resistance (MDR) mechanisms, the PTX can form a complex with Glutathione (GSH), thus passing or desensitization by stimulating expressions of resistance proteins, such as MRP2 or MRP1, P-gp. The increased dosage is a usual clinical approach for drug resistance regression, but high doses can cause toxicity in patients [6].

At present, combination therapy has received much attention in the medical field, especially in cancer treatment, because of its effectiveness in different ways. These ways include: (1) lowering drug doses to reduce the side-effects of high-dose PTX in patients, (2) delivery of drugs to the target site, (3) increasing anti-cancer efficiency, (4) reducing toxicity in normal cells, and (5) multiple drugs that can enhance the therapeutic activity [2, 7]. Recently, Talib, et al. [8] summarized the main combination therapy of the phytochemical compounds with chemotherapy, such as curcumin, resveratrol, genistein, epigallocatechingallate, thymoquinone, piperine, allicin, eparthenolide, modin, luteolin, quercetin, and anthocyanins and aspirin in co-application with vitamin D3 [9].

However, until now no study has reported the combination therapy of SS with PTX. Thus, the present study aimed to measure the anti-cancer activity and apoptotic effects of a new combination of SS with PTX at a ratio of 1:1 against liver cancer (HepG2) and normal cell lines (AML12) by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, fluorescent microscope, and cell cycle analysis (flow cytometry).

2. Materials and Methods

2.1. Materials

Several reagents and chemicals were used in the current study, including SS and PTX obtained from Stigma (Baoji-Guokang Bio-Technology Co., Ltd., China). The fluorescent probes DCFH-DA and (DAPI 4′-6-diamidino-2-phenylindole) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The HepG2 (human liver cancer) and AML-12 (normal human liver) cell lines were procured from IBRS, Tehran, Iran, and maintained in DMEM (Dulbecco's-Modified Eagle Medium) medium (Waltham, MA, USA, Gibco, Life Technologies) supplemented with fetal bovine serum (10%, BioWest S.A.S., Nuaille, France), sodium pyruvate (1%), and penicillin-streptomycin (1%).

2.2. Cell Seeding and MTT Assay

For cell seeding and MTT assay, cells were seeded into 96-well plates at a concentration of 5×10^3 cells/ml and incubated in a 5% CO_2 at 37ºC for 24 h. After incubation, the media was changed with new media containing SS and PTX alone and a combination of PTX with SS (at a ratio of 1:1) at different concentrations (ranging from 1000, 500, 250, 125, 62.5, and 0 µg/ml) and incubated for 72 h. MTT solution (20 µL) was loaded onto the cells and incubated for 4 h. We dissolved the formed formazan crystals in DMSO (dimethyl sulfoxide) and measured the absorbance at 570 nm using an ELISA reader. The cytotoxic activity of the SS, PTX, and PTX: SS was measured as the minimum inhibition concentration (IC_{50}) value. We calculated the cell viability percentage using the following formula:

$$Cellviability\ (\%) = Absorbanceofsample\ (mean) / Control\ (mean)\ x\ 100\ \%\ (1)$$

2.3. DAPI Staining

For DAPI staining with Fluorescent Microscope, the treated cells were fixed with glutaraldehyde solution (4%) and stained with DAPI before being photographed using fluorescence microscopy (USA, Invitrogen, Carlsbad, CA, EVOSTM FL).

2.4. Cell Cycle Analysis

For cell cycle analysis, after 24 h of incubation of treated cells (at a density of 5×10^5 cells/well), the cells were collected and washed once with phosphate buffered saline (PBS), adding 50 µL of cold PBS with gentle vortex. The cells were fixed with 7 % cold ethanol (1 ml), shaken for a few minutes to reduce cell clamping, and then stored in a refrigerator for 2 h. The ethanol was removed by centrifuging the cells and washed with PBS, the PBS was discarded. This was followed by re-suspension in PBS (950 µL) holding 40 µL of 1 mg/ml PI and 10 µL of 10 mg/ml RNase A. The cells were kept at room temperature for 30 minutes and subsequently analyzed using flow cytometry. Data were analyzed with FlowJo™ software.

2.5. Statistical Analysis

We conducted all analyses using GraphPad Prism software (version 5.0, USA). To determine significant differences between means, we utilized either the one-way Analysis of variance (ANOVA) or the unpaired Student's t-test. We produced the data at least three times and presented it as the mean plus standard deviation. The statistical significance level wasset at $P<0.05$.

3. Results and Discussion

3.1. Cytotoxic Effect of PTX and SS Single Application

SS (stigmasta-5,22-dien-3β-ol) is characterized by the presence of an isoprenyl tail and a hydroxyl group at position C-3 of the steroid skeleton and has double bonds at positions 5 and 6 of the B ring, and positions 22 and 23 in the alkyl substituent, as shown in Figure 1A[10]. PTX (tricyclic diterpenoid) is a compound consisting of three rings. It has a molecular formula of C47H51NO14 (Figure 1A).

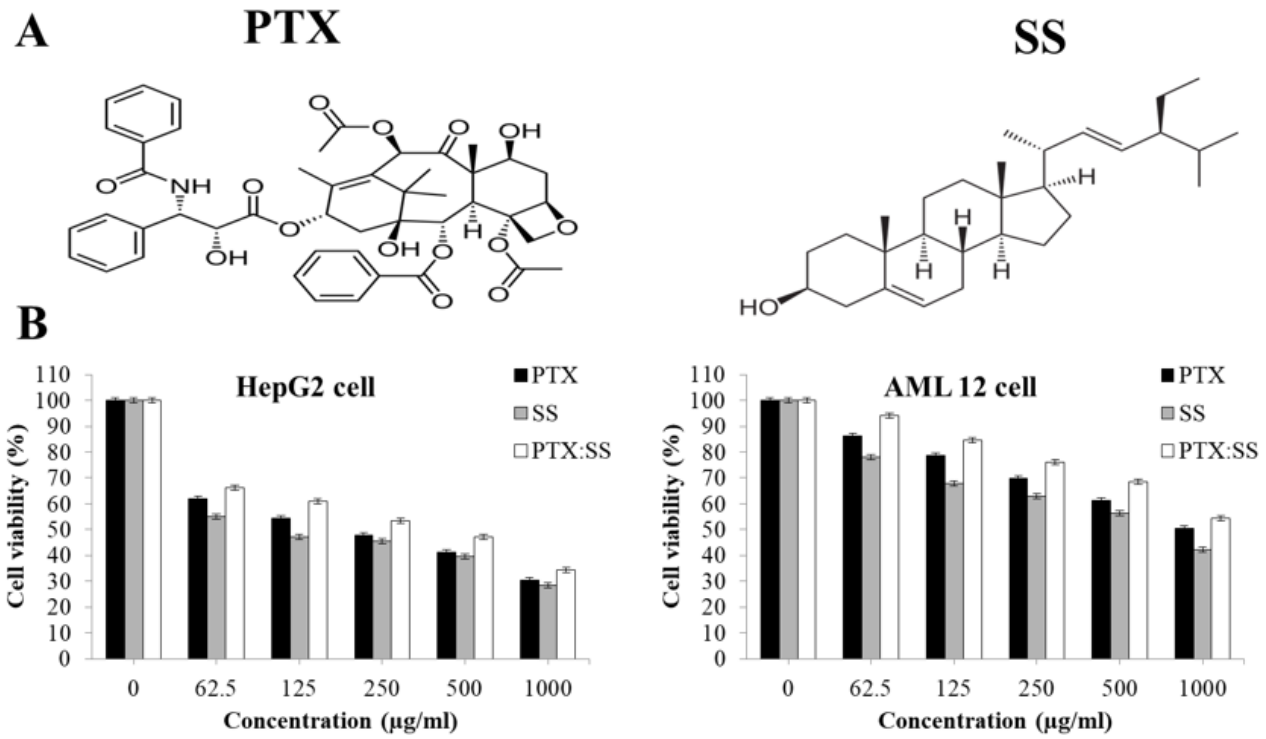

Figure 1.
Anti-proliferative effect of PTX, SS, and PTX: SS on HepG2 and AML cells. A) Chemical structures of PTX and SS, B) Cell viability of HepG2 and AML 12 cells after 72 h treatment with PTX, SS, and PTX: SS.

PTX ishighly hydrophobic and is thought to be part of the water-repellent region of the lipid bilayer of the cell memberane, which lets it get into the cytoplasm [11]. Cytotoxic effects of PTX and SS were assessed by the MTT assay after 72 h of incubation (Figure 1B and Table 1). The PTX and SS displayed a similar result because both had no cytotoxic effects on normal AML 12 cell lines, even at the concentration of 1000 µg/ml with IC_{50} of 933.53 and 728.49 µg/ml, respectively. For HepG2 cells, SS showed the highest cytotoxic effects (IC_{50} = 117.75 µg/ml) compared to PTX (IC_{50} = 294.59 µg/ml) and significantly reduced cell viability in HepG2 to 55, 47, 45, 39, and 28% at concentrations of 1000, 500, 250, 125, and 62.5 µg/ml, respectively, not affecting normal cells.

Table 1.
IC_{50} values (µg/ml) of PTX and SS in a free form and PTX: SS in combination form against HepG2 and AML12 cells.

Cell Lines	PTX	SS	PTX: SS (1:1)
HepG2	294.59± 0.56	117.75 ± 0.55	464.44 ± 0.34
AML 12	933.53 ± 0.41	728.49 ± 0.21	1056.44 ± 0.35

At concentrations of 500 and 1000 µg/ml, SS induced noteworthy cytotoxicity in the HepG2 cell line. Nonetheless, there have been studies that oppose this discovery and indicate that SS only exhibited weak activity against breast cancer MCF7 and MDA-MB-231 cells. This study found that even at the highest concentration, 70% of the cells remained viable, thus making it impossible to estimate the IC_{50}. Other studies described that SS compacts cell viability in a dose-dependent manner by inhibiting Akt/mTOR, JAK/STAT, and PI3K/Akt signaling pathways along with the VEGFR-2 pathway, arresting the G1 phase, inducing reactive oxygen species (ROS), inducting apoptosis through the caspase pathway, and others [10], as shown in Table 2.

Table 2.
Summary of the anti-cancer activity of SS against different cell-lines.

Anti-cancer compounds	Cell lines	Activity	References
Stigmasterol	Human umbilical vein endothelial cells (HUVECs)	Stigmasterol inhibiting cell viability, migration, and morphogenesis of human umbilical vein endothelial cells (HUVECs, IC_{50} 21.1 µM) but not cholangiocarcinoma (CCA) cells	Kangsamaksin, et al. [12]
Stigmasterol isolated from Naviculaincerta	Hepatocarcimona (HepG2) cells	Stigmasterol demonstrates potent apoptosis inductive effects and has the potential to be evaluated as an anti-cancer therapeutic against liver cancer.	Kim, et al. [4]
Stigmasterol	Human gastric cancer cell line SNU-1 and normal GES-1 cell line	The results confirmed that the stigmasterol showed higher cytotoxicity against gastric cancer (IC_{50} of 15 µM) with minimal activity on normal GES-1cells.	Li, et al. [13]
	Mouse model of DMBA-induced skin carcinoma	This study showed the anti-cancer activity with n antigenotoxic effect on DMBA-induced genotoxicity, confirming that stigmasterol's activity may be due to its antioxidant and antigenotoxic properties.	Ali, et al. [14]
	Human gastric cancer cell line SGC-7901, MGC-803, and normal GES-1 cell line	The results showed that stigmasterol significantly anti-cancer activity against SGC-7901 and MGC-803 cancer cells by inhibiting the Akt/mTOR pathway but did not stimulate the viabilities in normal GES-1, confirming that the effect of SS is specific to the gastric cancer cells.	Zhao, et al. [3]
Stigmasterol of the Mangrove plant Xylocarpusgranatum	Hela and MCF-7 cells	Stigmasterol showed good activity against Hela cells (123.2 µg/ml) as compared to MCF-7 cells	Rajeswari, et al. [15]
	MCF7 and MDA-MB-231	Stigmasterol showed a weak and no IC_{50} on MCF7 and MDA-MB-231 cells could be estimated because 70% cell viability was observed at the highest concentration.	Elshamy, et al. [16]
Stigmasterol isolated methanolic extract on leaves of *Crescentiaalata*Kunth	Hela cell line	The results show that the isolated stigmasterol has much potential in Hela (Mammalian cancer) cell line.	Abinaya [17]

A recent study revealed that the molecular modifications to the functional groups of SS can enhance its anticancer properties. The stigmasterol analogs demonstrated better cytotoxic activity overall than SS, which was found to be non-toxic to the triple-negative breast cancer (HCC70), MCF-7, and non-tumorigenic mammary epithelial (MCF-12 A) (EC50 > 250 µM). Stigmast-5-ene-3β,22,23-triol and 5,6-Epoxystigmast-22-en-3β-ol displayed improved cytotoxicity and selectivity against MCF-7 breast cancer cells with an EC50 values of 22.94 µM and 21.92, respectively, while stigmastane-3β,5,6,22,23-pentol showed enhanced cytotoxic activity against the HCC70 cell line with EC50 value of 16.82 µM[18].

However, the reasons for these differences between studies remain unclear, particularly when using the same cell line. In the current study, PTX alone seemed to have less impact against HepG2 cell lines than SS alone. Two potential factors can explain the present outcome. Firstly, HepG2 cells can effectively eliminate PTX at concentrations lower than 120 nM. Secondly, the presence of cytochrome P450 epoxygenase enzymes in HepG2 cells may explain their accelerated cell growth rate and greater resistance to PTX compared to HeLa-S3. These enzymes assist in breaking down PTX into less harmful metabolites, such as 6α-hydroxytaxol, which is 30 times less toxic than PTX [19, 20]. Further, apoptotic cells were identified through nuclear DNA fragmentation, while the viability of cells was determined by dye exclusion. It is worth noting that cells undergoing apoptosis are often scored as viable because disruption of the cell membrane typically occurs after DNA fragmentation. Therefore, we observed a significant increase in apoptosis in several cases, but no decrease in viability [19].

3.2. Cytotoxic Effect of PTX: SS Combination Application

Combining chemotherapy with SS is a promising direction for this study [21]. There were no significant differences observed for AML-12 cells after being treated with PTX: SS in relation to the control. Similarly, the combination of Bufalin and PTX did not show any effect on human normal cervical cells [22]. While the PTX: SS significantly impacted cell viability in HepG2 cells (34, 47, 53, 60, and 66 %, respectively) with IC_{50} of 464.44 µg/ml. Figure 2 summarizes the results, using a color code to differentiate the differences in relation to the control. In combination therapy for both cells, PTX: SS statistically boosted cell viability, differing from the single application, suggesting that SS may inhibit the effect of PTX in tested cell lines. This result confirmed a protective role of SS against PTX action and indicates that we are far from controlling, understanding, and expecting the real effects of SS over liver cancer cells. SS also suppresses chemoresistance and enhances the inhibitory activity of some anticancer drugs Bakrim, et al. [10]. Elshamy, et al. [23]

reported that a combination of SS and sorafenib may be an effective therapeutic regimen for treating breast cancer by modulating crosstalk between ERK/Caspase-3 and NF-κB-VEGF/BCL-2 signaling axes [23].

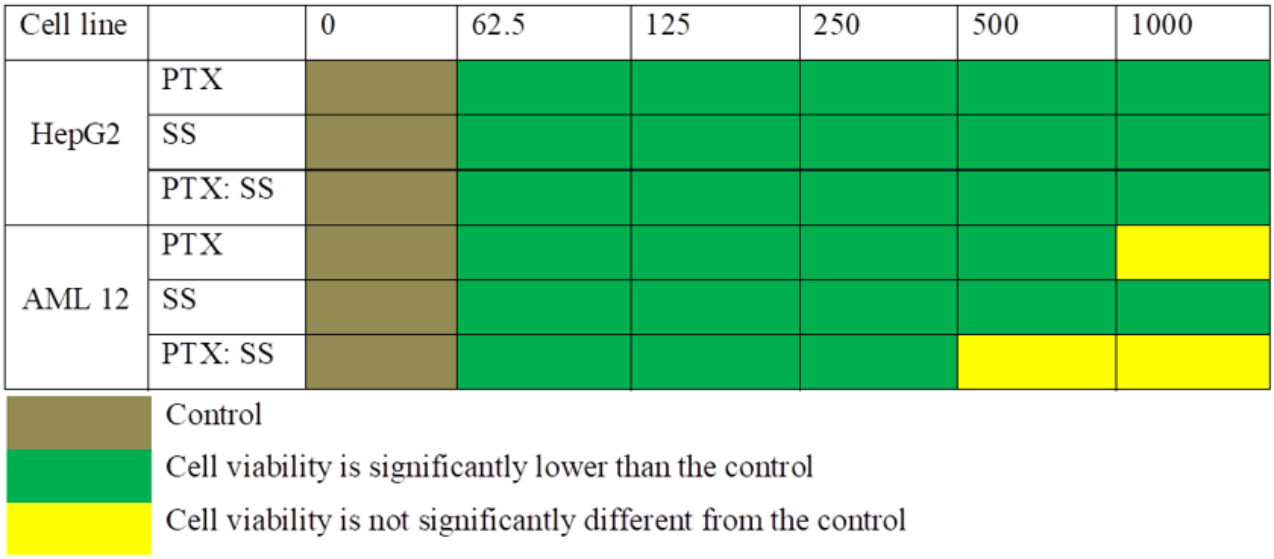

Figure 2.
Summary of the cell viability results of the combination SS compound and PTX drugs after 72 h treatment.

To our knowledge, no studies exist regarding the combination of SS and PTX on HepG2 and AML-12 cells. The response of cancer cells to treatment may depend on the concentration of SS and PTX and the type of cancer cell line. PTX combined with SS did not enhance the overall cytotoxic performance against HepG2 cell lines. However, this study found that PTX: SS could reduce the side effects arising from the treatment with PTX. In fact, SS may provide additional cytotoxic effects to the target tissue because of its higher concentration, resulting in improved effectiveness of combined therapy.

However, regarding the combination therapy of PTX with bioactive compounds, our results align with previous studies where a combination of 2.5 nM PTX with 2.5 µM tetracycline-3 (COL-3) resulted in a small reduction in cell viability as compared to COL-3 alone. However, these results go against what other research had found, which was that 5-Demethylnobiletin (5-DMN) enhanced PTX activity on CL1–5 (lung cancer) cells by inducing apoptosis through the Caspase pathway. Additionally, the authors confirmed in an in vivo study that treatment with 5-DMN and PTX significantly reduced tumor growth in a nude mouse xenograft model [24]. Further, curcumin combined with PTX (CUR-PTX) showed a significant reduction in the IC_{50} value compared to PTX alone. This combination led to increased apoptosis in CD44+ prostate cancer stem cells, and the expression of CD44 and P-gp was significantly decreased after being treated with CUR-PTX. CUR-PTX resulted in higher levels of miR-148a, which helped regulate the levels of its target genes (IRS1 and MSK1). It was also discovered that CUR might increase PTX sensitivity by increasing miR-148a expression and impacting its target genes [25-27]. Differently, combining Curcumin with PTX enhanced cytotoxicity in HepG2 cells by down-regulating Lin28 (RNA-binding protein) [28].

3.3. DAPI Staining

The DAPI (fluorescent dye) binds to the DNA's minor groove (A-T region). At higher concentrations of PTX, SS, and PTS: SS (1000 µg/ml), morphological changes occur in cells, leading to the induction of apoptosis. These changes include cell shrinkage, chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies. Control (untreated) cells showed healthy cells with blue nuclei and maintained intact mitochondrial membranes [29]. On the other hand, the HepG2-treated cells showed chromatin condensation of cell nuclei in a dose-dependent manner, DNA fragmentation, and decreased cell nuclei, suggesting that SS, PTX, and PTX: SS could induce HepG2 cell apoptosis (Figure 3A). The AML-12 normal-treated cells exhibited no significant morphological changes compared to HepG2 cells (Figure 3B).

We got the same results as other studies that showed SS could cause apoptosis in HepG2 cells through a normal apoptotic pathway, especially when the cells were treated with a lot of SS [4]. The DAPI staining results showed that SS prompts apoptosis in gastric cancer (SNU-1) cell lines with distinctive morphological changes, such as cell shrinkage, cell blebbing, and nuclear damage [13]. PTX alone showed a considerably increased count of fragmented nuclei in AGS gastric cancer cells in comparison to untreated cells [30]. Also, the SKOV-3 cells (ovarian adenocarcinomas) that were treated with PTX and piperine lost their membrane integrity, which proved that cyt-c had been released from the mitochondria. The combined treatment resulted in reduced DNA damage and mitochondrial membrane potential, which strongly indicates chromatin condensation and reduced the potential of mitochondrial membrane [29].

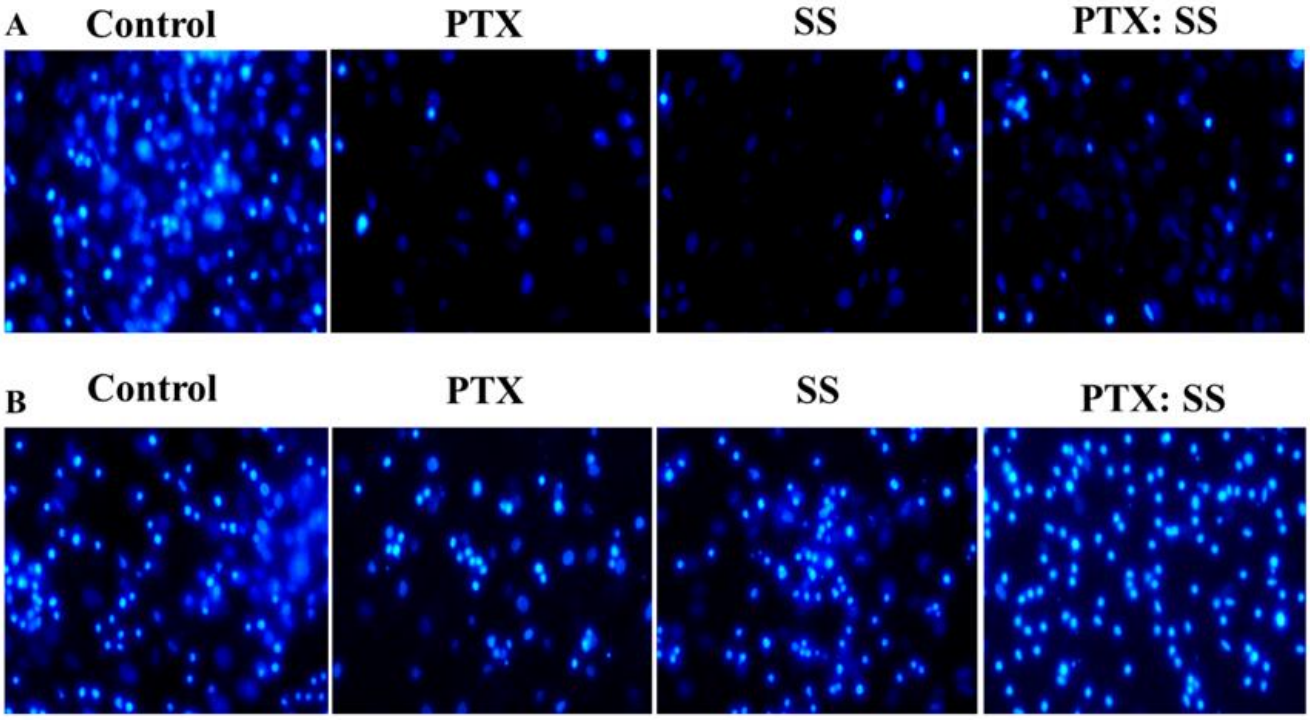

Figure 3.
The DAPI stained cell morphology was detected by fluorescence light microscope: (A) HepG2 cells and (B) AML 12 cells.

3.4. Cell Cycle Analysis

Cyclins and cyclin-dependent protein kinases regulate the cell cycle, an essential process of cell reproduction closely related to cell proliferation [31]. Cell-cycle checkpoints are key data for determining the cell death procedure in cancer cell-lines. There are four major phases in cell cycle analyses, including sub-G1, G0/G1, and S phase, which are essential checkpoints in cell apoptosis. Thus, any DNA damage results in a stop to proceeding to the next generation and cell death [4, 32, 33]. The determined cell phase population cells treated with PTX, SS, and PTX: SS were disclosed using flow cytometry analysis (Figure 4).

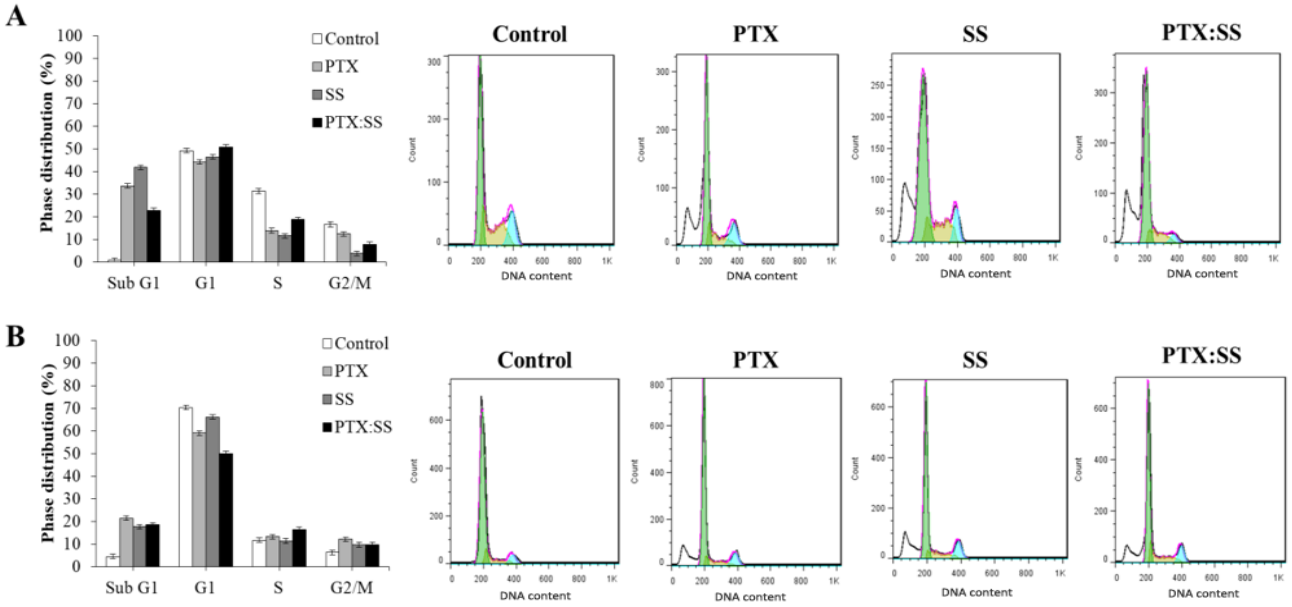

Figure 4.
The cell cycle progression patterns of HepG2 (A) and AML 12 (B) cells treated with PTX, SS, and PTX: SS after stained with PI and detected using FACS.

Figures 4A and 4B show how the different stages of cell cycle are spread out in the HepG2 and AML12 cell lines after being treated for 24 h.We observed an increase in sub-G1 phase distribution for HepG2 cells (41.76, 33.64, and 22.83%) with SS, PTX, and PTX: SS treatment, respectively, compared with the control (0.72%), confirming that the SS treatment induced higher apoptosis compared to PTX alone and combined therapy. The results showed a significant decrease in S phase (18.73, 13.8, and 11.53%) and G2/M phases (7.73, 12.29, and 3.69%) after being treated with PTX: SS, PTX, and SS, respectively, compared to control (16.6%). As shown in Figure 4, PTX, SS, and PTX: SS caused a comparable decrease in the G1 phase in a dose-dependent manner against both HepG2 and AML 12 cells. For AML 12 normal cells, the highest

sub G1 phase was observed with PTX (21.4%), SS, and PTX: SS treatment (4.49, 17.64, and 18.42%, respectively), with a comparable increase in the S and G2/M phases compared to the control. However, the previous study demonstrated a dose-dependent increase in the G0/G1phase cell population in AN3CA and EMC6 endometrial cancer cells treated with SS for 48 and 72 h, compared to the untreated cells. This was achieved by decreasing the levels of cyclin D1, CDK6, and CDK2, and increasing the activity of retinoblastoma (RB) by reducing its phosphorylation. On the other hand, the population of S phase decreased in the SS treatment group. These results conclude that SS significantly causes cell cycle arrest at the G1 phase in EC cells [34]. Cells should lose enough DNA to appear in the sub-G1 phase. Thus, the cells that do not appear in sub-G1 undergo apoptosis in the S or G2/M phase of the cell cycle. Further, defects in cell components led to cell cycle arrest, causing damaged cells to undergo apoptosis [35]. These results suggest that the SS treatment resulted in the HepG2 cell cycle arrest, leading to apoptosis and cell death, but not in normal healthy cells. Our results aligned with a previous study confirming that HepG2 cells were arrested at the G2/M phase of the cell cycle after being treated with SS [4]. According toZhang [36] SS can stop liver cancer cells from growing by inducing cell arrest in the G0-G1 phase, resulting in fewer cells in the G2/M phase. The study also noted an increase in the protein expression of protein kinase MAP2K6, an important protein in cell cycle arrest. The previous study showed that PTX alone caused a significant increase in sub-G1 phase of A375 cells (53.83%), while not affecting the G2/M phase. In addition, cells treated with PD98059 did not show any effect on the sub-G0/G1 phase (3.04%) compared to the untreated control, but resulted in a significant increase in the percentage of cells in the G0/G1 phase (82.59%). The combination of PD98059 and PTX resulted in a further increase in the population of apoptotic cells (79.52%) [37].

4. Conclusion

This study showed synergistic cytotoxic effects of the combined SS bioactive compounds and PTX drug on HepG2 and AML-12 cell lines for the first time. These results confirmed the significant activity of the SS alone on HepG2 without affecting normal healthy cells compared to PTX treatment, which causes cytotoxic effects against normal cells. In combination therapy, cell viability significantly decreased relative to control but did not differ statistically from the SS and PTX alone. DAPI staining and cell cycle analyses have confirmed these results. The evaluations revealed that SS and PTX induced more apoptotic events at sub-G1, but SS treatment specifically caused the cell cycle arrest at the G2/M phase compared to PTX: SS treatment. The research findings reveal that SS holds promise in combating cancer cells in the HepG2 cell line and can also alleviate the adverse effects of PTX on normal cells. However, establishing an effective synergistic application is a complex process that relies on various factors such as the cell type, compound concentration, and their interactions. Therefore, further investigation is necessary to explore alternative methods of delivering both SS and PTX and determine their optimal concentrations. Moreover, obtaining a comprehensive understanding of the molecular mechanisms of this combination therapy is crucial. Preclinical and clinical trials are imperative to verify the drug combination's efficacy and safety in humans.

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