Medical Science

25(114), August, 2021

Evaluation of anticancer activity of Paclitaxel (PLT) and D-L Sulfarophane (SFN) as natural vegetable derivative against colon cancer cell line: In vitro study

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To Cite:
Ali AHA, Almalki AM, Alharbi TA, Alanzy MH, Alghuwainem MA, Alanazi FM, Alshumaymiri NI, Mohamed AF. Evaluation of anticancer activity of Paclitaxel (PLT) and D-L Sulfarophane (SFN) as natural vegetable derivative against colon cancer cell line: In vitro study. Medical Science, 2021, 25(114), 1900-1909

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Peer-Review History
Received: 26 June 2021
Reviewed & Revised: 27/June/2021 to 24/July/2021
Accepted: 25 July 2021
Published: August 2021

Peer-review Method
External peer-review was done through double-blind method.

ABSTRACT

Paclitaxel (PTx) and D-L Sulforaphane (SFN) natural derivatives could inhibit many types of cancerous cells proliferation through apoptosis and autophagy. Toxicity of PTx and SFN to Caco-2 cells was concentration dependent using MTT assay. Sole SFN was significantly toxic than sole PTx. PTx and SFN combination induced a synergetic potential of PTX toxicity than sole form and the recorded IC50 in µgm / ml was in the order of (862.8,2.,8 and 22.37) for sole PTx, SFN and combined form respectively. PTx and SFN sole and combined form induced Caco-2 cells DNA accumulation at the G2/M phase but not significantly at the Go/G1 and S phases. Apoptosis was proved via a significant up regulation of P53, Cy-c, Bax, casp-3 and down regulation of Bcl-2 with elevated Bax/Bcl-2 ratio. Antioxidant activity supported apoptotic potential showed significantly elevated ROS, MDA and reduced GSH.

Keywords: Anticancer, Paclitaxel, Sulfarophane, Cytotoxicity, Apoptosis, Flow cytometry, Cancer colon

1. INTRODUCTION

Paclitaxel (PTX) as anticancer biotherapy has a well-known potential via interfering with microtubule dynamics (Steinmetz & Prota, 2018). However, recent reports the PTX therapy induced anticancer resistance increased and promoted metastasis (Volk-Draper et al., 2014). Combination therapy has been shown to reduce drug resistance, dose and toxicity to achieve the synergistic effects of anti-cancer agents (Kendra et al., 2015). Sulforaphane (SFN) derived

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from cruciferous vegetables is a good substance to suppress many types of cancers. SFN united with PTX was showed to encourage PTX-induced cell death (Burnett et al., 2017; Kim et al., 2017). SFN form sulforaphane-N-acetyl-cysteine (SFNNAC) and sulforaphane-cysteine (SFN-Cys), those were plentiful in plasma and lungs compared with SFN (Clarke et al., 2011). SFN metabolites can suppress proliferation of cancer and autophagy (Hu et al., 2018), inducing apoptosis (Zhou et al., 2018).

Anticancer activity of SFN may be attributed to isothiocyanates, produced of myrosinase-mediated glucosinolate degradation. Sulforaphane (SFN) is isothiocyanate derivative from special type of vegetables (cruciferous), like cabbage and broccoli. Efficacy, safety, minimal drawbacks, and affordability, bioactive SFN is widely recognized as a promising anticancer agent including different cancer types (Abdull Razis & Noor, 2013; Cheng et al., 2016; Peng Cheng et al., 2015; Leone et al., 2017; Juengel et al., 2016; Wang et al., 2017; Clarke et al., 2008). Also, SFN has been revealed to promote chemotherapeutic cisplain activity (Wang et al., 2016). Analysis of cell cycle showed that SFN induce DNA accumulation at the G2/M phase inhibiting of tumor proliferation, associated with down regulation of cyclin B1 (Cheng et al., 2016) and cyclin D1 genes (Żuryń et al., 2016), and increased protein levels of p21WAF1/CIP1 inducing depletion of cyclin-dependent kinases (Żuryń et al., 2016). To stimulate apoptosis in cancer cells; SFN increased the pro-apoptotic protein Bax expression and decreased expression of the anti-apoptotic protein Bcl-x (Kim et al., 2015). SFN inhibited the angiogenesis and metastasis of ovarian cancers by suppressing the activity and expression of hypoxia inducible factor-1α (HIF-1α) (Pastorek et al., 2015; Kim et al., 2015). Exposure to oxidative stress for long time promotes carcinogenesis that encourage damage DNA, inflammation and mutation (Narendhirakannan & Hannah, 2013; Kensler et al., 2012; Johnson et al., 2017; Liu et al., 2015). Many studies showed that the effects SFN on Nrf2 through alteration of Keap1 cysteine remains (Hu et al., 2011) stimulation of mitogen-activated protein kinase (MAPK), and other factors (Magesh et al., 2012; Qin & Hou, 2016; Zhang et al., 2013; Su et al., 2014).

The present work aimed to evaluate the anticancer potential of naturally derived pTx in its sole form and combined form with SFN and related biochemical, apoptotic genes and cell cycle profiles, histopathological altrations and biochemiaal profile.

2. MATERIALS AND METHODS

Paclitaxel and D-L Sulforaphane

Paclitaxel 6mg/ ml and D-L Sulforaphane (SFN) 5 mg/ ml were purchased from (Sigma - Aldrich, USA) I dark bottles in a lyophilized form, D-L Sulforaphane was dissolved in DMSO(ICI-UK)

Colon cancer cell line

Human colon cells (Caco-2) were purchased from tissue culture department (The holding company for production of vaccine, sera and drugs (VACSERA, Giza, Egypt). The cells were kept in RPMI 1640 media at 37°C in incubator with 5% CO_2. Cells were preserved according to the instructions of manufacturer.

In vitro cell viability (MTT assay)

The effect of Paclitaxel , slufaphuran (SFN) and paclitaxel-SFN mix Caco-2 cell viability was determined using MTT assay, where Caco-2 cells were seeded in 96-well plates (Costar®, Corning, Switzerland) as 1 × 104 cells/well and incubated for 24 hours at 37°C in a humidified incubator in 5% CO2 atmosphere (Jouan, France). The growth medium was replaced with 100 µL/well of two fold serially diluted Paclitaxel, slufaphuran (SFN) and paclitaxel-SFN mix in serum-free medium. After 24 hours of treatment, the treatment medium was decanted and cells were washed using (PBS) Phosphate buffer saline (ADWIA –Egypt) as 250 µL /well in triplicate. MTT solution 50 µL/well of the (0.5 mg/mL in PBS), were dispensed to the whole plates followed by incubation for 3 to 4 hours at 37°C. The supernatants were carefully removed, and the crystals of formazan were dissolved with dimethyl sulfoxide using 50µL/well (Signma –Aldrich, USA). The plates were gently shacked for 15 minutes at 37oC. Absorbance was determined at 570 nm with a microplate reader (Bio Tek, Winooski, VT, USA). The percentage of viability was calculated using the following formula:

Viability (%) = (A570 of treated cells / A570 of control cells) × 100.

Analysis of cell cycle

Distribution of cell cycle was tested by estimating the content DNA of nuclei marked with propidium iodide (PI). Au-NPs electroporated and ELFEMF treated Hep-2 cells were pelleted by cold centrifugation (Jouan Ki-21-France), washed with 1 ml of PBS, in ethanol at +4°C for 24 hrs. Then, cells were processed with RNase A (20 mg/ml) and PI (20 mg/ml), FITC conjugated Annexin-V for 30 min at 37°C in darkness. Finally, analysis of cell cycle distribution was carrying out using flow cytometry.

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Quantitative real-time-PCR

Total RNA was removed from control, and paclitaxel, SFN and paclitaxel-SFN mix IC50 treated Caco-2 cells using RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. The extracted RNA concentration of was estimated using Beckman dual spectrophotometer. Reverse transcription was performed using thermal cycler (Applied Biosystems) at 37oC; Step 3, 5 minutes at 85°C; then Step 4, hold at 4°C. The expression level of related genes-apoptosis: P53 (R: 5′-GGG TGT GGA ATC AAC CCA CAG-3′ and F: 5′-TCA GAT CCT AGC GTC GAG CCC-3′), BAX (F: 5′- R: 5′-CCC AGT TGA AGT TGC CGT CA-3′ ATG GAC GGG TCC GGG GAG CA-3′ and BCL2 (F: 5′-GTG AAC TGG GGG AGG ATT GT-3′ and R: 5′-GGA GAA ATC AAA CAG AGG CC-3′) and CYC (F: 5′ AGTGTTCCCAGT GCCACACCG-3′) R: 5′ TCCTCTCCCCAGAATGATGCC TTT-3′ Casp-3 (F: GGAAGCGAATCAATGGACTCTGG and (R: GCATCGACATCTGTACCAG and housekeeping gene; ACTB (F 5′AGCGAGCAT CCCCCAAAGTT-3′ and R: 5′-GGGCACGAAGGCTCA TCATT-3) were determined using real-time PCR. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of B-actin as house keeping gene by calculating the delta cycle threshold. The primers were manufactured by LGC Biosearch technologies (Novato, CA, USA).

Biochemical analysis

Caco-2 IC50 treated cells (floating and adherent cells) were washed with cold PBS (by centrifugation), and cold centrifuged (Jouan-Ki22, France) pelleted at 1500 RPM for 15 minutes. The cell lysate was prepared by re- (Jouan, Ki-22, France) suspending the cell pellet in a cold PBS, followed by sonication in ice path and cold centrifuged at 4,000 ×g for 15 minutes at 4°C. The supernatant was used for assay. The MDA, [ROS] Reactive Oxygen Species and glutathione reductase [GSH] activity levels were, performed according to instructions of the manufacturer's, assayed in treated and untreated cells using readymade kits provided by Biodiagnostic (Cairo, Egypt) and Milton Roy spectronic 21D UV-Visible spectrophotometer (USA).

Statistical analysis

All experiments were carried out independently at least three times. The IC50 values were determined using Graph Pad Prism 7 (GraphPad software, La Jolla, CA, USA). The P values were gained from comparing the control group versus each treatment group using unpaired Student t-test. The differences were considered statistically significant at $P < 0.05$.

3. RESULTS

Cell culture (MTT assay)

Cytotoxicity of test products in sole and mix forms indicated that cytotoxicity was concentration and drug type dependent as viability as increasing as long as the concentration decreased. Also, SFN showed a significantly reduced IC50% value (P<0.05) compared with its value in case of paclitaxel, while the use of mixed drugs showed synergetic activity of paclitaxel toxic effect recording a significantly reduced IC50 value (P<0.05) (Fig. 1 A-D).

Data recorded revealed that there was a significant (P<0.05) elevation of pro-apoptotic genes (P53) compared with its value in untreated cell control. In the meantime there was a significant elevated apoptotic genes Bax, Cy-c and casp-3 post cell treatment with Paclitaxel, SFN and Paclitaxel-SFN mix and control respectively and compared with untreated cell control. Similarly there was a significant down regulation of anti- apoptotic gene (Bcl-2) (Fig. 2). From the presented data the effect of SFN was significantly effective on apoptotic gene profile than that of paclitaxel and paclitaxel-SFN mix.

Concurrently apoptotic gene profile was in accompanied with change in the cell cycle profile, where the DNA accumulation post cellular treatment with paclitaxel, SFN and the combined form revealed that there was an insignificant (P>0.05). DNA accumulation during the Go/G1 phase (34.69%,32.2%,34.39%), while it recorded (25%, 35.19%, 35.44%) during the S phase and (28.17%, 22.36%, 18.63%) during the G2/M phase post Caco-2 cells treatment with SFN, Paclitaxel and the paclitaxel – SFN mix respectively compared with its values detected in case of untreated cell control recording (52.99%,36.74%, 8.06%). DNA content during G2/M phase was significantly elevated than in case of cell treatment with sole paclitaxel and paclitaxel –SFN mix (P<0.05). Finally there was a significant elevated total apoptosis post cell treatment with sole paclitaxel, SFN and their mix compared with control (12.14%, 10.25%, 11.54%). With significantly elevated Early and Late apoptosis in case of cell treatment with SFN, sole paclitaxel and Paclitaxel-SFN, mix compared with control values. Finally necrotic activity was significantly elevated (P<0.05) post cell treatment with SFN than in case of sole Paclitaxel and paclitaxel – SFN mix (Fig. 4A- B).

Biochemical alteration induced post cellular treatment with paclitaxel, SFN and the combined form showed that there was a significant (P<0.05) decreased GSH level in case of Caco-2 cells treatment with SFN than that in case of cell treatment with sole paclitaxel and paclitaxel- SFN combined form Also, treatment forms showed a significant (P<0.05) decreased GSH than that of

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untreated cell control Use of Paclitaxel –SFN mix showed insignificant decreased GSH than in case of paclitaxel treated cells (Fig 3A-D). Similarly lipid peroxidation represented by MDA level it was noticed that SFN, Paclitaxel sole and paclitaxel-SFN combined form showed a significant (P<0.05) elevated MDA level compared with its level of untreated cell control. ALSo, SFN induced a significant elevated MDA level than in case of sole paclitaxel and paclitaxel – SFN mix, while the combined form was not significantly elevated than in case of sole paclitaxel (Fig 4A- B). In the mean times, reactive oxygen species (ROS) level showed significant elevated values compared with untreated cell control value. In addition, SFN showed a significant elevated ROS values than sole paclitaxel and paclitaxel- SFN mix (P<0.05). Also, paclitaxel – SFN mix showed insignificant elevated ROS than in case of cell treatment with Sole paclitaxel (Fig.5A-B, Fig 6 A-D).

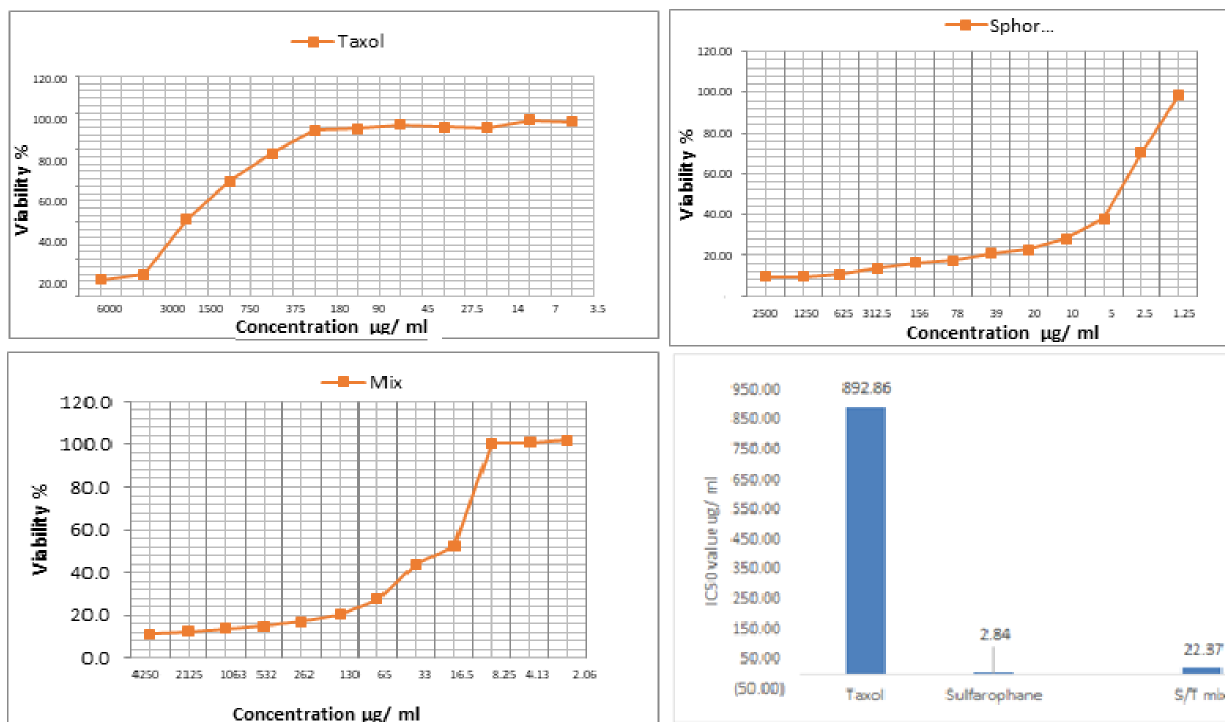

Figure 1A-D Evaluation of CAco-2 cells viability post treatment with paclitaxel, SFN and combined mix using MTT assay

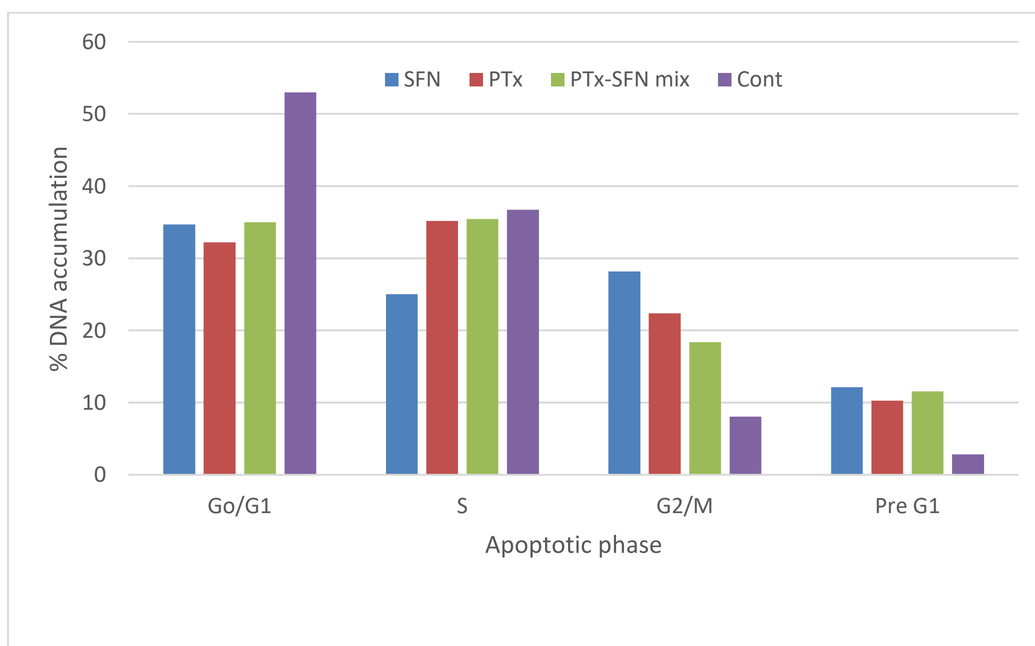

Figure 2 Evaluation of apoptotic and anti-apoptotic gens post Caco-2 treatment with Paclitaxel, SFN and Paclitaxel-SFN mix compared with cell control.

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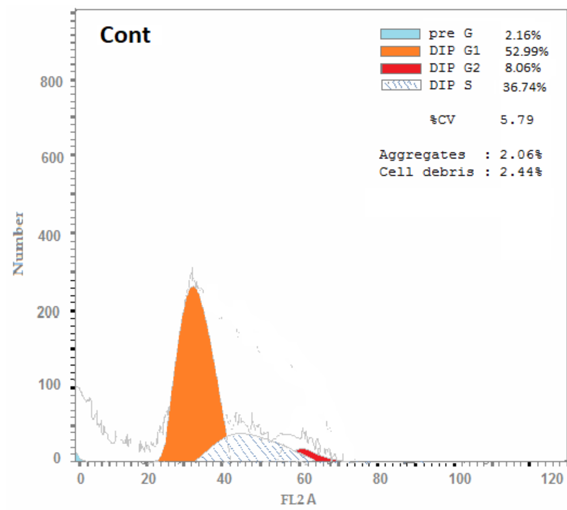

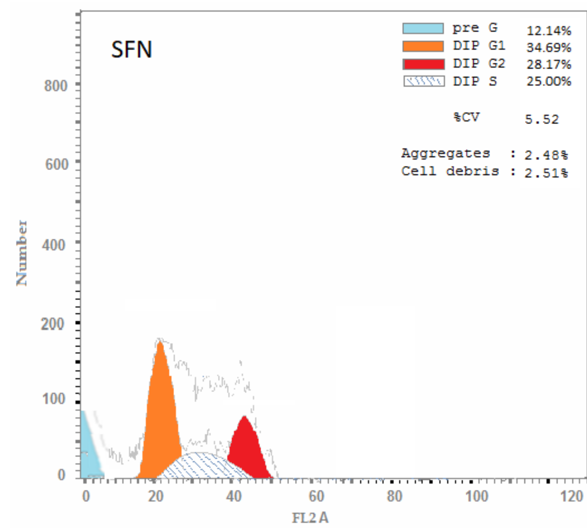

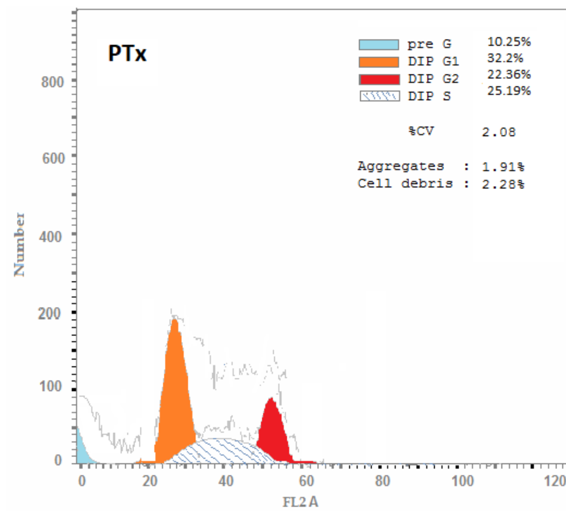

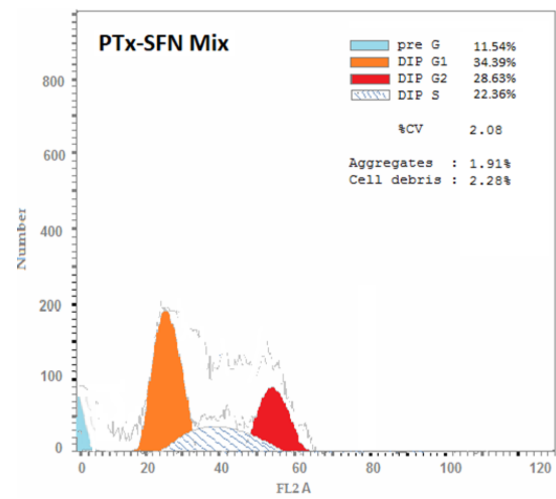

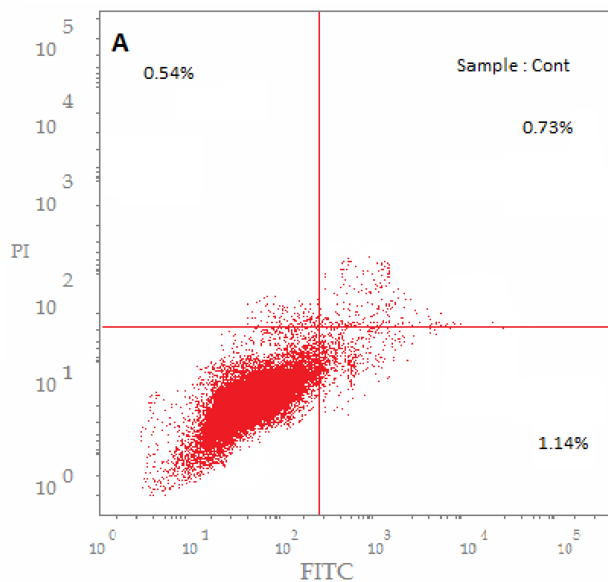

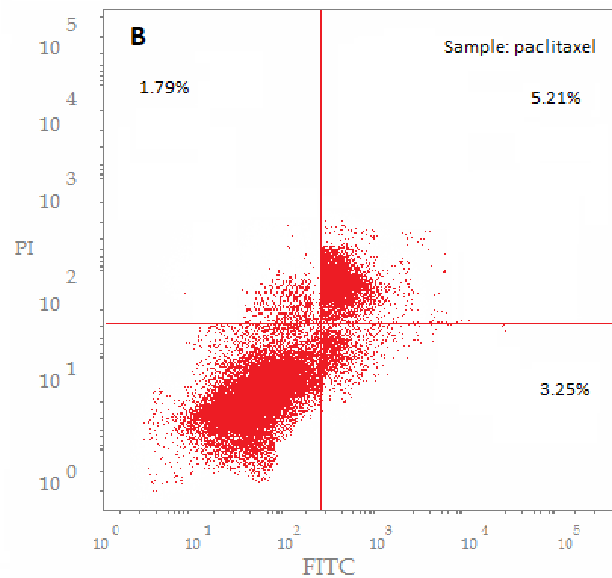

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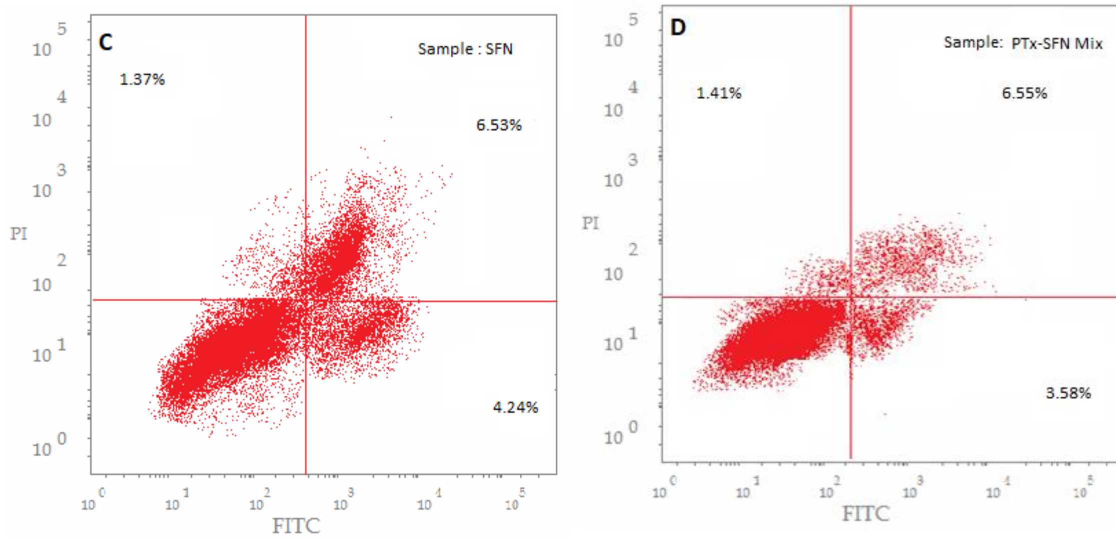

Figure 3A-D Evaluation of DNA content and cellular apoptosis post Caco2 with Paclitaxel, SFN and Paclitaxel-SFN mix using flow cytometry analysis.

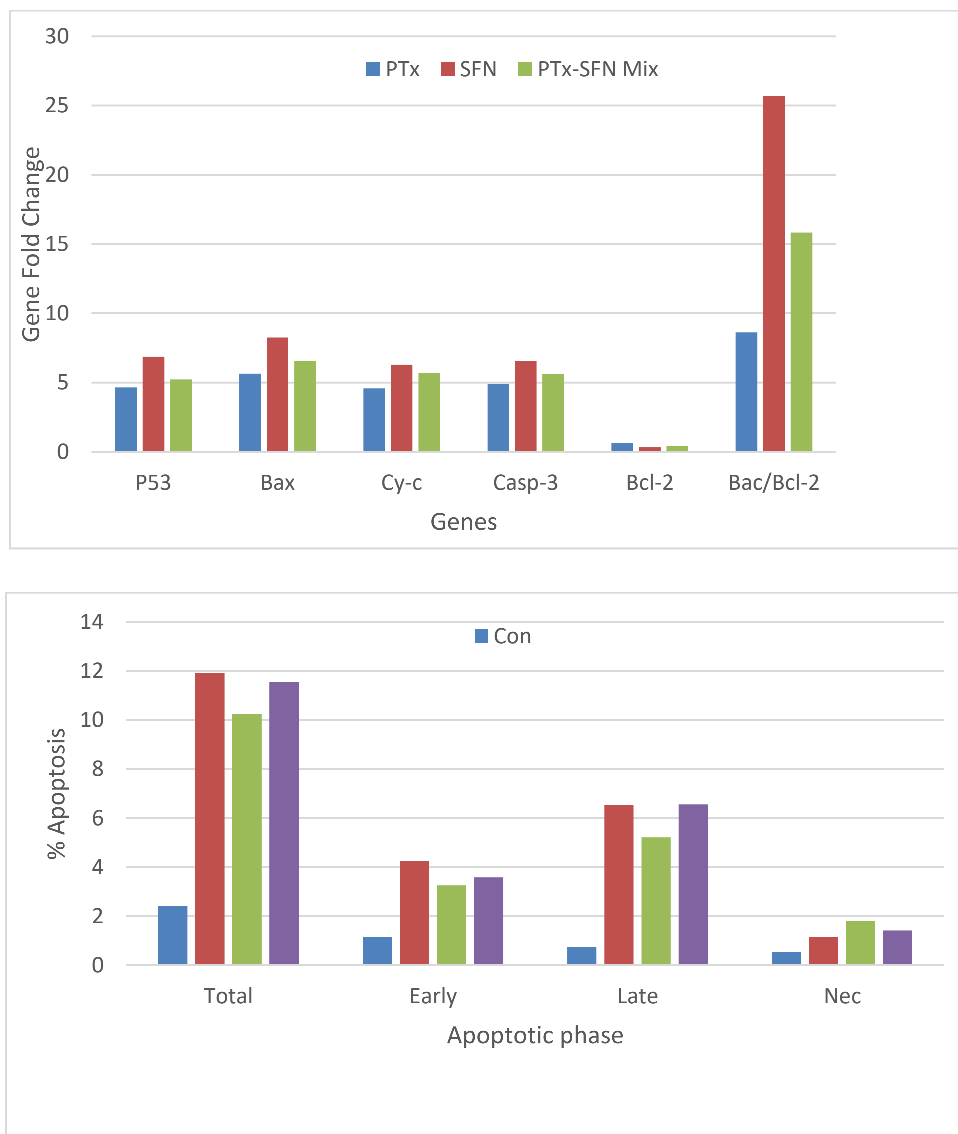

Figure 4 A-B Evaluation and DNA content and cellular apoptosis post Caco2 with Paclitaxel, SFN and Paclitaxel-SFN mix using flow cytometry analysis.

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2.5
2
1.5
1
0.5
0
GSH ng / ml
PTx SFN Mix Cont
Tretament
GSH

200
150
100
50
0
ROS pg/ml
PTx SFN Mix con
Treatment
ROS

A **B**

3
2.5
2
1.5
1
0.5
0
MDA µ mol/ml
PTx SFN Mix con
Treatment
MDA

C

Figure 5 A-C Evaluation of ROS, GSH and MDA post Caco-2 cells treatment with paclitaxel, SFN and combined form using biochemical assay pathology.

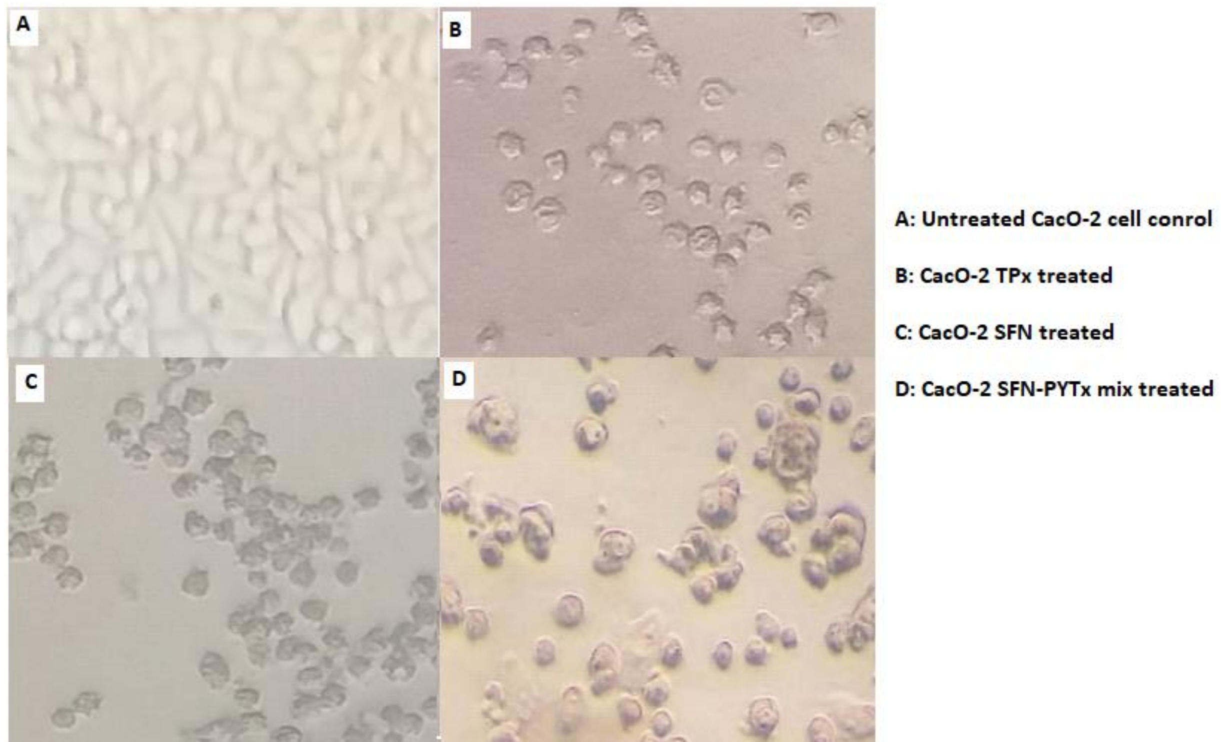

Figure 6 A: Untreated CacO-2 cell control, B: CacO-2 TPx treated. C: CacO-2 SFN treated. D: CacO-2 SFN-PYTx mix treated.

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4. DISCUSSION

Some studies reported that the main cause of resistance production is overexpression of class III microtubule associated proteins, microtubule stabilization protein Tau, and nti-apoptotic protein X-linked inhibitor of apoptosis protein (XIAP) (Abdull Razis & Noor, 2013). Increased expression of βIII-tubulin promoted cell survival and drug resistance to PTX in NSCLC cells (Kim et al., 2015; Narendhirakannan & Hannah, 2013). XIAP is mainly as a potent suppressor through blocking Caspase-3-mediated apoptosis (Kensler et al., 2012).

Development of drug resistance resulted due to elevated XIAP that related to cancer drug resistance (Johnson et al., 2017; Liu et al., 2015). Resistance to anticancer drug may be induced due to Tau protein that encourages microtubule stabilization and tubulin assembly, and may attach to the inner surface of the microtubule (Hu et al., 2011). It was found that high expression of Tau promoting to the chemical resistance to PTX. Also, over expression of Hsp70 may be in charge of tumor progression through supplying resistance to chemotherapy (Su et al., 2014). It was found that SFN metabolites induced α-tubulin degradation and microtubule disruption through ERK1/2 phosphorylation (Wang et al., 2016), and SFN-mediated upregulation of 26S proteasome through sustained ERK1/2 phosphorylation that lead to cell apoptosis and microtubule disruption (Gerhauser, 2013; Meeran & Ahmed, 2010).

5. CONCLUSION

It can be concluded that both PTX and SFN showed anticancer activity against Caco- 2 cells SFN is extremely toxic than PTX. And PTx and SFN combination sowed synergetic potential of PTx cytotoxicity efficiently as that of sole SFN.

Recommendations

It can be recommended that, PTX application must be used in mixed form with SFN. Nanocapsulation of both sole SFN and Mixed SFN-PTx for long term sustained release must be evaluated. *In Vivo* study with Histopathological and survival activity must be conducted.

Acknowledgments

This publication was supported by the Deanship of Scientific Research at Prince Sattam bin Abdulaziz University, Alkharj, Saudi Arabia. In addition we thank those who participated and contributed to the study.

Authors' Contributions

All authors contributed to the research and/or preparation of the manuscript. Ali Hassan A. Ali, Aly Fahmy Mohamed and Abdulrahman M. Almalki participated in the study design and wrote the first draft of the manuscript. Talal A. Alharbi, Musaad H. Alanzy, Nasser I. Alshumaymiri and Fahd M. Alanazi collected and processed the samples. Muath A. Alghuwainem participated in the study design and performed the statistical analyses. All of the authors read and approved the final manuscript.

Funding

This study has not received any external funding.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval

All series of steps that were implemented in this study that included animal models were in compliance with Ethics Committee of Prince Sattam bin Abdulaziz University Institutional Review Board (PSAU-2020 ANT 1/41PI).

Data and materials availability

All data associated with this study are present in the paper.

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