



Antiproliferative and apoptotic activities of 8-prenylnaringenin against human colon cancer cells

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ABSTRACT

Aims: The compound 8-prenylnaringenin (8-PN) is a prenylflavonoid that can be isolated from hops and beer and has anti-cancer properties against breast cancer. The aim of this study is to investigate the anti-proliferative and apoptotic activities of 8-PN against human colon cancer HCT-116 cells.

Main methods: Colon cancer HCT-116 cells were treated with 8-PN and subjected to MTT and acridine orange/propidium iodide (AO/PI) staining to investigate the cytotoxicity of 8-PN. Arrest of the cells at different phases of cell cycle was monitored in the presence of 8-PN. Moreover, the apoptotic effects of 8-PN was assessed via annexin V and caspase activity assays and compared to the untreated cells.

Key findings: The findings showed that 8-PN revealed strong inhibitory effect against HCT-116 cells with an IC_{50} value of $23.83 \pm 2.9 \mu\text{g/ml}$ after 48 h. However, at similar concentrations and experimental time-points, the compound did not show cytotoxic effect to non-cancerous colon cells (CCD-41). Annexin-V assay indicates that 38.5% and 14.4% of HCT-116 cells had entered early and late stages of apoptosis, respectively after exposure of the cells to 8-PN for 48 h. Caspase activity assay illustrates that apoptosis is activated through both intrinsic and extrinsic pathways. Moreover, flow cytometry cell cycle results indicate that treatment with 8-PN significantly arrested the HCT-116 cells at G0/G1 phase.

Significance: These findings reveal that 8-PN has anti-proliferative activity against HCT-116 colon cancer cells via induction of intrinsic and extrinsic pathway-mediated apoptosis. Further investigations should be carried out to unravel the mechanistic pathways underlying these activities.

1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer in the world [1]. In 2012, approximately 1.36 million new cases were diagnosed with CRC, out of which 694,000 deaths were reported [2]. Although CRC is more common in developed countries, mortality rate is higher in developing countries [1,2]. Modern lifestyle has been associated with higher rates of obesity, diabetes, ageing, consumption of alcohol and cigarettes, and also lack of physical activity, all of which can contribute to increased risk of CRC [3–5]. It is predicted that the incidence of CRC will be increased by 90% in 2030 [6]. The current management of colon cancer such as surgery, radiotherapy and chemotherapy have considerable side effects including cytotoxicity in other organs such as heart, kidney, bladder, lung and the nervous system. As an example, 5-fluorouracil (5-FU), which is commonly used to treat different types of colon cancer, is associated with a wide range

of adverse effects, such as cardiovascular toxicity, diarrhea, dehydration, abdominal pain, nausea, stomatitis and hand-foot syndrome [36–40]. Taking all these into consideration, there is an urgent need for new chemotherapeutic agents to combat CRC with minimal toxicity to normal tissue and possess a more favorable therapeutic window.

Apoptosis induction is an important target for cancer treatment. Apoptosis is a programmed cell death in which proteolytic enzymes called caspases play an important role in this event. Activation of caspase cascades is usually initiated by either intracellular or extracellular signals through activation of apoptosis stimulators. The release of cytochrome C from the mitochondria activates caspase 9, while fas-associated protein with death domain (FADD) cleaves pro-caspase 8 to the active form leading to cell death [7–10]. It is to be noted that this event is usually regulated by anti-apoptotic proteins such as Bcl-2 and inhibitors of apoptosis protein (IAP) family [11–14]. Ideally, an agent that is able to promote cancer cells to undergo apoptosis could be

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selected as a promising candidate to combat cancer.

In the last few decades, research has focused on various flavonoid compounds, which had demonstrated potential pharmacological properties. These compounds have shown beneficial health effects against various diseases including cancer [15,16]. The compound 8-prenylaringenin (8-PN) is a prenylflavonoid found in different natural sources such as, hops (*Humulus lupulus*) and beer. The flowers from hops are used in beer making, which provide the bitter flavor and pungent aroma of beer [17]. It has been shown that hops extract has anti-proliferative properties against some colon cancer cells such as HT-29 and SW620. Moreover, the anti-proliferative effects of hops extract are due to the xanthohumol and isoxanthohumol and are enriched in hops. Same as 8-PN, xanthohumol and isoxanthohumol are other members of prenylflavonoid [18]. Despite various studies that had been carried out on different flavonoids, studies assessing the anticancer properties of 8-PN is still very limited.

Increasing evidence suggests that 8-PN can trigger MAP kinase pathway and induce apoptosis in MCF-7 breast cancer cells [19]. Additionally, a study by Pepper et al. [20] have also demonstrated that 8-PN inhibited angiogenesis in bovine microvascular endothelial (BME) and endothelial cells from the bovine thoracic aorta (BAE) cells at a concentration of 3–10 μM [20]. Another study reported that 8-PN demonstrated a biphasic action on MCF-7 breast cancer cells; whereby the compound induced cell proliferation at 10^{-8} – 10^{-6} M concentration, but on the other hand apoptosis was observed to happen at 10^{-5} M concentration [19]. Furthermore, 8-PN acts as a potent inhibitor of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and may be used as a chemopreventive compound against colon and liver cancer [21–23]. In addition, 8-PN inhibits cell viability of colorectal Caco-2 cells at concentration of 40–50 μM and Caco-2 cells were arrested in G0/G1 phase [24]. Despite these evidences, the effect of 8-PN against some important colorectal cell lines such as HCT-116 has not yet been investigated. Therefore, this study was designed to assess the pro-apoptotic properties of 8-PN against HCT-116 colon cancer cells using in vitro assays and to identify the possible apoptotic pathway using caspase cascade and annexin V assay.

2. Methodology

2.1. Compound and cell lines

The compound 8-prenylaringenin (Fig. 1A) was purchased from Santa Cruz Biotechnology, USA with molecular weight of 340.37 g/mol and purity > 98% (lot # A3014). Colon cancer (HCT-116) and normal colon (CCD-841) cell lines were purchased from ATCC, USA, whereas Dulbecco's modified Eagle's medium (DMEM/high glucose) was obtained from HyClone, USA. Fetal Bovine Serum (FBS) and penicillin-streptomycin were purchased from Biowest, France.

2.2. Cell viability assay

Cell viability of HCT-116 (colon cancer cells) and CCD-841 (normal colon cells) were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25]. Cells with density of 5000 cells/well were seeded in 96 transparent plates and incubated overnight at 37 °C. Cells were treated with different concentrations of 8-PN with the highest concentration of 100 $\mu\text{g}/\text{ml}$ and serial dilution factor of 2 for 9 concentrations. The anticancer drug 5-fluorouracil (5-FU) was used as a positive control. Three wells in each plate were treated with the solvent (0.02% DMSO) and served as negative control. Cells were incubated for 24, 48 and 72 h at 37 °C in 5% CO_2 . Subsequently, 20 μl of MTT was added to each well and further incubated for 4 h. Following the incubation process, the solution was aspirated and 100 μl of DMSO was added into each well. The cytotoxicity effect of 8-PN against HCT-116 and CCD-841 cells was measured using microplate reader (Tecan, Infinite M1000) at 570 nm. Data were calculated as

percentage of cell inhibition using the following formula:

$$\text{Cell inhibition (\%)} = \frac{\text{sample OD} - \text{control OD}}{\text{control OD}} \times 100$$

Cell viability assay was performed in triplicates in three independent experiments. The concentration of 8-PN with 50% cell growth inhibition was defined as the IC_{50} value, which was determined through use of a linear plot.

2.3. AO/PI assay

The viability state of HCT-116 cells was further assessed by using acridine orange (AO) and propidium iodide (PI) fluorescent dye [26,27]. Briefly, 10^6 cells were seeded in 25 cm^2 tissue culture flask and treated with the IC_{50} of 8-PN and incubated at 37 °C for 24, 48 and 72 h. Untreated flask was kept as a control. After that, cells were detached and washed with cold phosphate buffered saline (PBS) for three times. After centrifugation, the cell pellets were suspended in 100 μl of PBS, 10 $\mu\text{g}/\text{ml}$ of AO and 10 $\mu\text{g}/\text{ml}$ of PI. Fluorescence inverted microscope (Nikon, Japan) was used to detect the morphological changes of the cells in which green color stained cells represent normal or cells at early stage of apoptosis, while necrotic cells are stained with red color.

2.4. Cell cycle

Flow cytometry was used to determine the distribution of HCT-116 cells at different phases of the cell cycle in the presence of 8-PN. Content of DNA in the cells was detected with propidium iodide (PI) staining of HCT-116 cells using flow cytometry measurements [28]. Briefly, cells were seeded in a well plate at a density of 10^6 cells in a complete medium and were treated with 8-PN for different periods of time (24, 48 and 72 h). Negative control was treated with 0.02% DMSO. The cells were collected and fixed with 70% ethanol at -20 °C overnight. Cells were washed and stained with 500 μl of PI/RNase staining buffer (Becton Dickinson, USA) and then incubated for 30 mins at room temperature. Each experiment was conducted in triplicates. Cell cycle phase distribution was determined using BD FACSCanto II flow cytometer instrument and BD FACS Diva software (Becton Dickinson, USA). Totally 15,000 events per sample were recorded for analysis. ModFit LT version 3.0 software was used for analyzing the data.

2.5. Annexin V/PI assay

Annexin V assay was used to study the cellular apoptosis of the treated and untreated cells. Conjunction of propidium iodide (PI) with annexin V helps to determine if the cells are dead, apoptotic or viable due to their cell membrane permeability and integrity [29,30]. In this assay 10^6 HCT-116 cells were seeded in a 6-well plate with complete medium at 37 °C overnight. Cells were treated with 8-PN at different time points (24, 48 and 72 h). Then, cells were washed with cold PBS and suspended in $1 \times$ binding buffer at the concentration of 1×10^6 cells/ml. A 100 μl volume of this suspension was transferred to 6 ml polystyrene round-bottom FACS tubes and incubated with 5 μl of PI and 5 μl of FITC-Annexin V for 20 min at room temperature. Subsequently, 400 μl of $1 \times$ binding buffer was added to this suspension and introduced to the FACS Caliber instrument (Becton Dickinson, USA) to investigate the apoptosis.

2.6. Caspase activity assay

Caspase-Glo 3/7, Caspase-Glo 8 and Caspase-Glo 9 (Promega, USA) are luminescence based assays which were used to evaluate the effect of 8-PN on caspase cascades in treated HCT-116 cells [22]. Briefly, white plate (SPL, Korea) containing 1×10^4 cells/well was incubated at 37 °C overnight. The IC_{50} of 8-PN (23.83 $\mu\text{g}/\text{ml}$) was used to treat the cells for 24, 48 and 72 h. This assay was performed in triplicates for each period

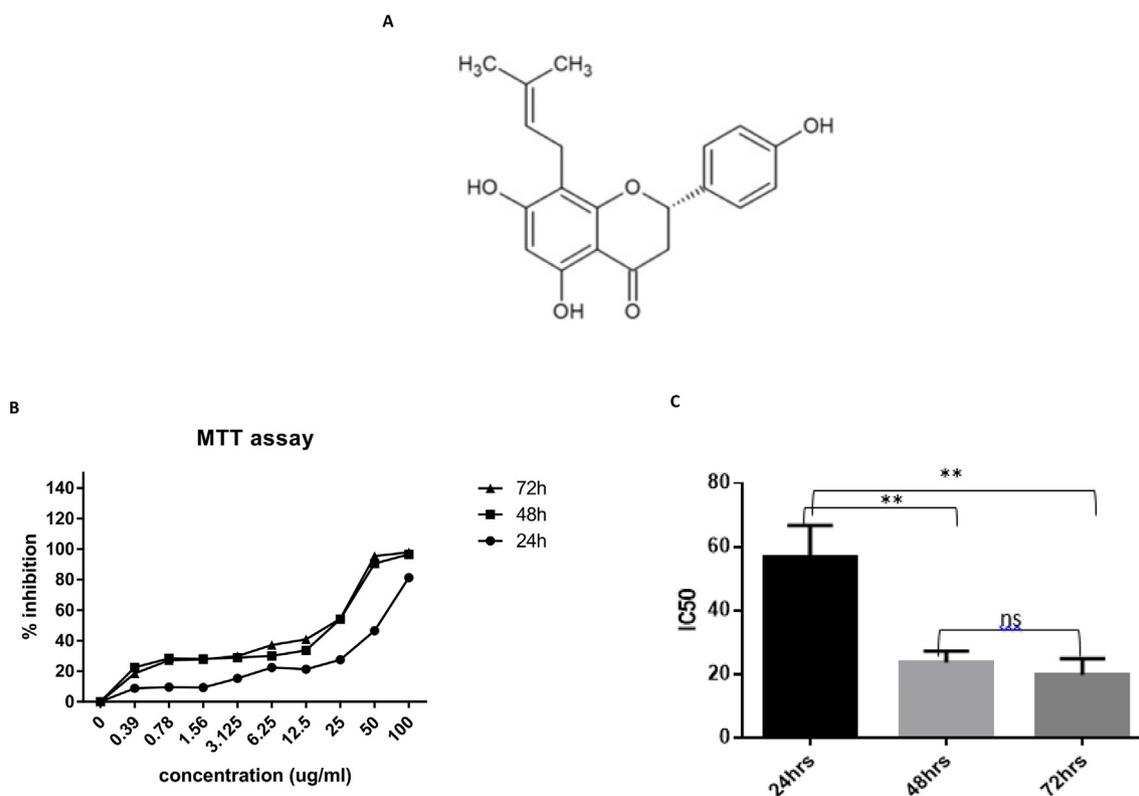


Fig. 1. (A) 8-Prenylnaringenin chemical structure. (B) Cell inhibition percentage of HCT-116 treated cells with 8-PN at different time points. (C) The IC₅₀ of 8-PN against HCT-116 cells after 24 h, 48 h and 72 h. The IC₅₀ of 8-PN against HCT-116 cells after 24, 48 and 72 h was determined to be 56.67 µg/ml, 23.83 µg/ml and 19.91 µg/ml, respectively. Data were expressed as mean ± standard deviation. **p < 0.01, indicates significant differences compared to control.

of time. Another 6 wells were preserved for samples with no cells but with media and with reagents as the blank samples, and for the untreated cells as negative control. At the end of the incubation period, 100 µl of caspase-Glo reagent contain substrate, buffer and MG-132 inhibitor were added to each well and mixed gently by using plate shaker at 300–500 rpm for 30 s. The plate was incubated in the dark at room temperature for 1 h and then the optical density was measured via luminescence microplate reader (Promega, USA).

2.7. Statistical analysis

All the data were expressed as mean ± standard deviation (SD) of three replicates. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons. Data were analyzed with SPSS (Statistical Package for the Social Sciences), version 21.0 for windows. The values of p < 0.05 were considered statistically significant.

3. Results

3.1. Cell viability

HCT-116 cell line is one of the most common epithelial colorectal carcinoma cell lines in which the cells were isolated from adult male with colon cancer and are ideal for in vitro study. In this study the cytotoxic effect of 8-PN against HCT-116 cells was carried out using MTT assay. Treatment with 8-PN inhibited the growth of HCT-116 cells in a time dependent manner and the IC₅₀ for 8-PN against HCT-116 was 23.83 ± 2.9 µg/ml and 19.91 ± 4.1 µg/ml after 48 and 72 h, respectively (Fig. 1B & C). On the other hand, 8-PN at this concentration did not show cytotoxic effect against CCD-841 normal colon cells, which indicates the high selectivity of 8-PN against cancer cells. The inhibitory activity of 5-fluorouracil (5-FU) against HCT-116 cells served

as a positive control. The IC₅₀ of 8-PN after 48 h (23.83 ± 2.9 µg/ml) was significantly lower than the IC₅₀ of 8-PN after 24 h (56.67 ± 8.2 µg/ml).

3.2. AO/PI double staining

AO/PI fluorescent staining was applied to observe the morphological changes related to apoptosis induced by 8-PN after 24, 48 and 72 h of treatment [31]. Fig. 2A shows the morphological changes of cell death in the presence and absence of 8-PN. In untreated cells (negative control) the nucleus was intact and most of the cells were viable (green color). Upon treatment with 8-PN, cells started to have blebbing, which indicates that cells had entered the apoptotic phase. Binding of AO and PI stains to the DNA of the cells generated greenish-orange color for the cells in the early stage of apoptosis. Gradually, when the cells entered the late apoptosis phase, the greenish-orange color of the cells turned to a reddish-orange color until the cells entered the necrotic phase in which the morphology of the cells changed to being red, swollen and enlarged. Fig. 2A shows that in the presence of 8-PN the percentage of cells which entered the apoptotic and necrotic phases had increased in a time dependent manner.

3.3. Induction of cell cycle arrest at G₀/G₁ phase in HCT-116 cells by 8-PN

Cell cycle analysis was conducted to determine the effect of 8-PN on cell cycle disruption after 24, 48 and 72 h after treatment using PI dye. Fig. 2B & C demonstrated that the number of the cells were accumulated in G₀/G₁ phase at each time point from 24 to 72 h and cell numbers in S and G₂ phases were decreased in a time-dependent manner. This suggests that HCT-116 cell cycle arrest had occurred at the G₀/G₁ phase in the presence of 8-PN.

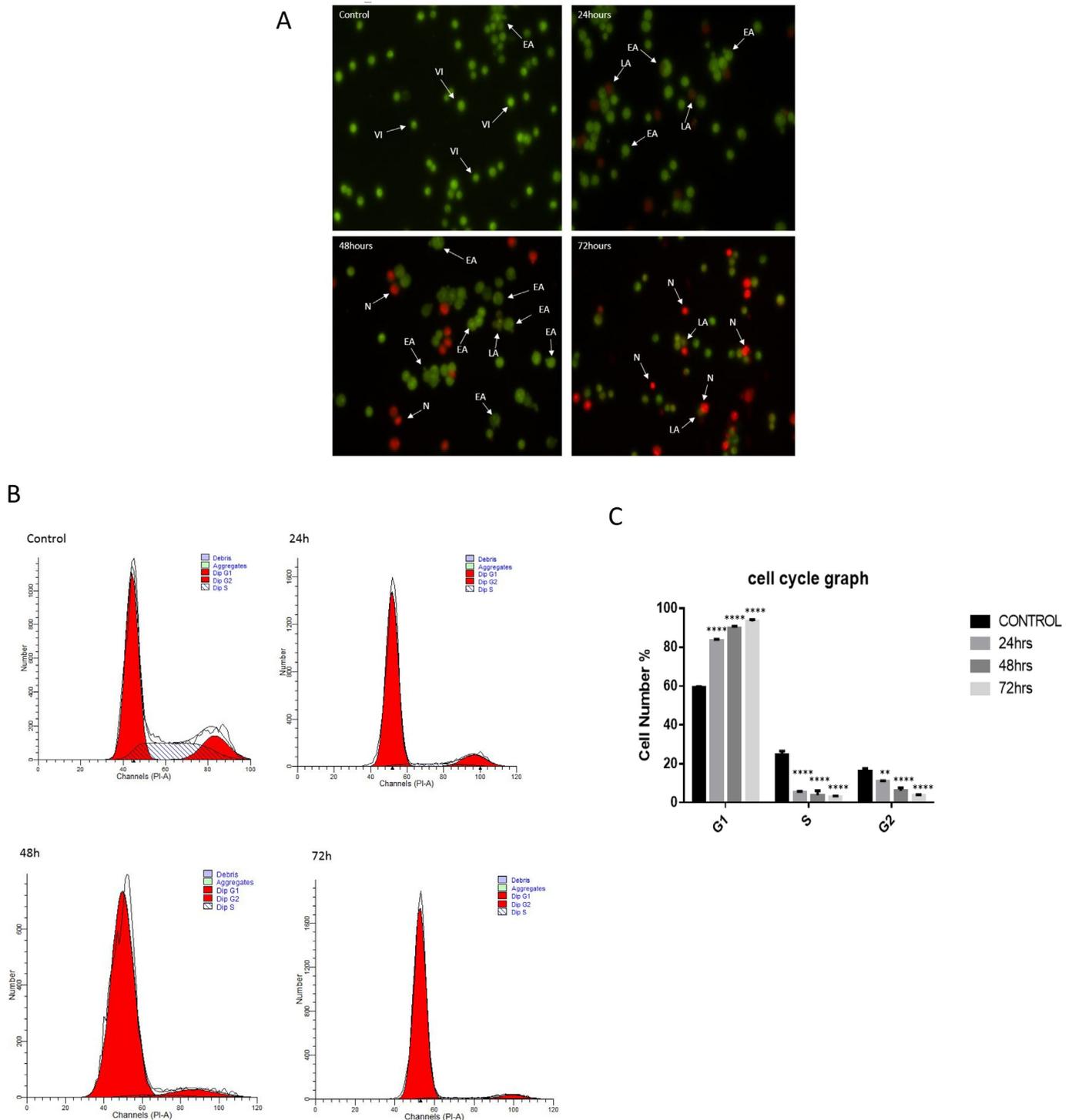


Fig. 2. (A) AO/PI staining of HCT-116 cells (control and treated cells with 8-PN after 24, 48 and 72 h). Percentage of viable cells reduced in the presence of 8-PN and cells entered to the apoptosis phase gradually. AO: acridine orange; PI: propidium iodide; EA: early apoptotic cells; LA: late apoptotic cells; N: necrotic cells; VI: viable cells. (B) Effect of 8-PN on disruption of HCT-116 cell cycle. After treating cells with the IC₅₀ of 8-PN for 24, 48 and 72 h, flow cytometric analysis was performed for treated cells and control. (C) Quantitative analysis of the effect of 8-PN on disruption of HCT-116 cell cycle. Quantitative analysis demonstrated that cells were arrested at G₀/G₁ phase. Data were expressed as mean ± standard deviation. **p < 0.01, ****p < 0.0001 indicate significant differences compared to control.

3.4. Annexin V

Annexin-V FITC kit was used for this experiment. HCT-116 cells were stained with annexin-V/PI after exposing them to 23.83 µg/ml of 8-PN for 24, 48 and 72 h. Results revealed from flow cytometric analysis indicate that cell apoptosis occurred in a time dependent manner (Fig. 3A). In the untreated cells (control), about 89.8% of cells were

viable, while HCT-116 cells exposed to 8-PN for 24, 48 and 72 h showed reduction in the percentage of viable cells (ranged from 58.7% to 28.5%). The percentage of treated cells in early stage of apoptosis reached 26.8% after 24 h of treatment and this percentage further increased to 38.5% and 59.1% after 48 h and 72 h of treatment, respectively. On the other hand, the percentage of the treated cells in late apoptosis phase were in the range of 12.2%–14.4%, which indicate the

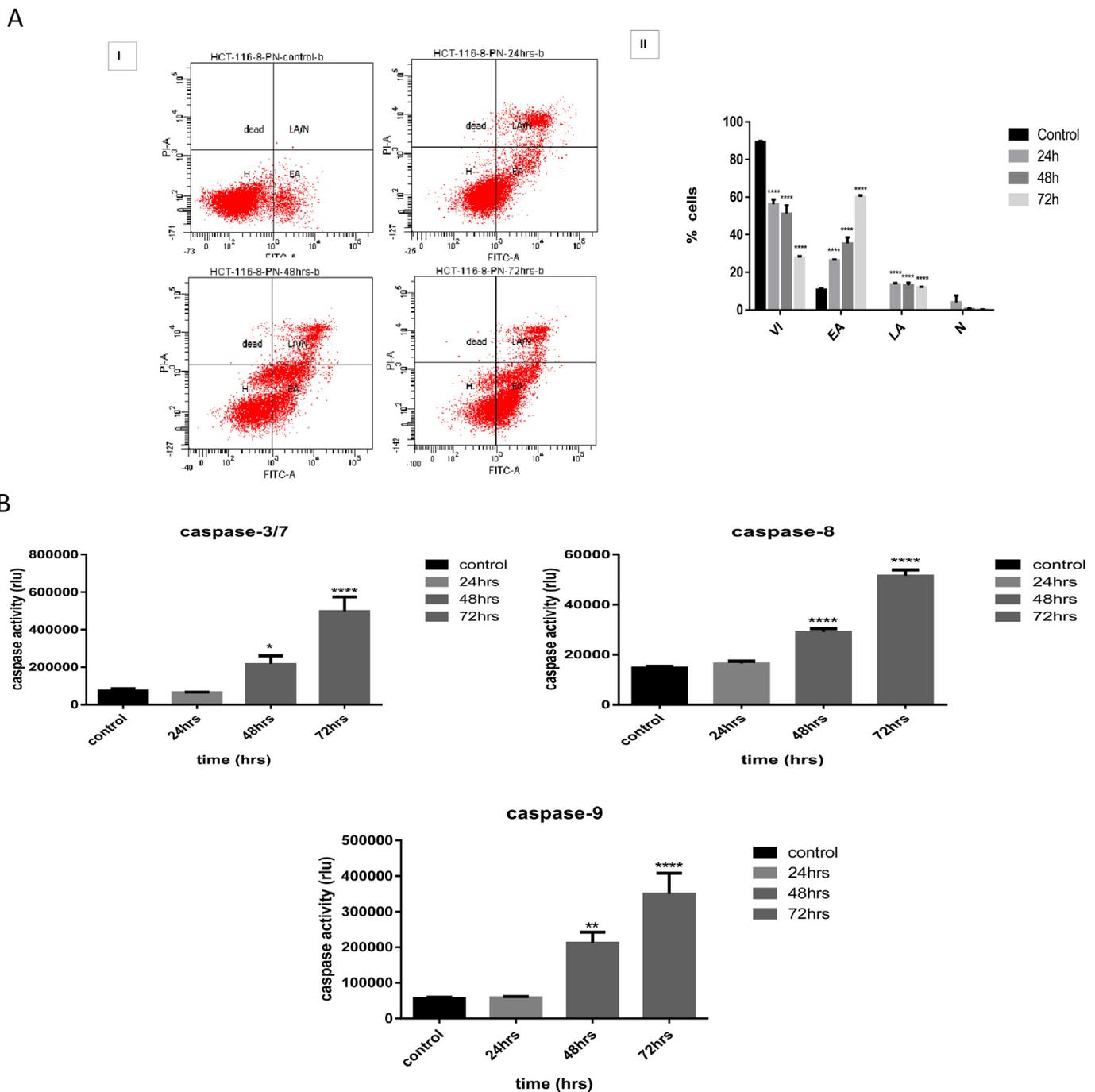


Fig. 3. (A) Flow cytometric analysis of Annexin V in HCT-116 cells in absence and presence of 8-PN at different time points. I: The distribution of the cells in different stages, II: The percentage of the cells in each stage. VI: viable cells; EA: early apoptosis; LA: late apoptosis; N: necrosis. (B) Caspase activity assay of 8-PN in HCT-116 cells. The luminescence analysis demonstrated a significant difference in expression level of caspases 3/7, 8 and 9 after 48 and 72 h. Data were expressed as mean \pm standard deviation. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ indicate significant differences as compared to control.

dramatic increase in late apoptosis phase as compared to the untreated control cells (0%). This result suggests that apoptosis occurred in a time-dependent manner in 8-PN-treated cells.

3.5. Caspase activity

In this study, caspase activity of 8-PN was measured using the caspase Glo kit. Pro-caspase was used as substrate for luciferase activity in which its cleavage results in generation of chemiluminescence signal. It is to be noted that in this experiment, the intensity of luminescence produced by pro-caspase cleavage is proportional to the amount of

caspase activity of treated cells.

As shown in Fig. 3B, our results indicated that apoptosis was activated through both intrinsic and extrinsic pathways following treatment with 8-PN at 48 h time point. Activation of caspase cascades was not significantly altered in the 8-PN-treated cells after 24 h as compared to the untreated control cells. However, after 48 h of treatment, the amount of caspase cascades increased significantly as compared to the control and this trend was observed until 72 h of treatment. Since caspase-8 and 9 activities had significantly increased in the 8-PN treated cells as compared to the control, this result indicates that both extrinsic and intrinsic pathways of apoptosis were activated after 48

and 72 h of treatment.

4. Discussion

Overall, our MTT results showed a dramatic reduction of HCT-116 cell viability in the presence of 8-PN after 48 h treatment with an IC_{50} of $23.83 \pm 2.9 \mu\text{g/ml}$. On the other hand, the same concentration of 8-PN did not show any cytotoxic effect against CCD-841 (normal colon) cells, which indicates the selective anti-proliferative effects of 8-PN compound. In our study we investigated the cytotoxic effect of 8-PN at different time points, whereas previous studies mostly carried out at 24 h of treatment only. It seems that endothelial cells ($IC_{50} = 3\text{--}10 \mu\text{M}$) and MCF-7 breast cancer cells (10^{-5}M) are the most sensitive cells to 8-PN followed by Caco-2 cells ($IC_{50} = 40\text{--}50 \mu\text{M}$) [19,20,24], whereas in our study the IC_{50} of 8-PN against HCT-116 cells after 24 h of treatment was higher (IC_{50} : $56.68 \mu\text{g/ml} = 66.5 \mu\text{M}$) compared to other cell lines. Interestingly, the IC_{50} of 8-PN reduced to 23.83 and 19.91 $\mu\text{g/ml}$ after 48 and 72 h of treatment, respectively.

Cancer development is usually characterized by abnormal cell proliferation. Mutation in any of the genes that are involved in the regulation of cell cycle or growth factor signaling pathway can lead to cell signal autonomy [32]. Our cell cycle results indicate that the anti-proliferative properties of 8-PN is due to the potential activity of 8-PN to arrest HCT-116 cells at G0/G1 phase of cell cycle. Allsopp et al. [24] have been reported that Caco-2 colon cancer cells treated with 8-PN were arrested at G0/G1 phase, which is similar to our findings, however for MCF-7 breast cancer cells, arrest in the presence of 8-PN occurred at S phase due to the inhibition of Cyclin D [19]. The discrepancy in the findings between studies indicates that cell cycle arrest properties of 8-PN is cell specific and might vary from one cancer cells to another.

Beside the anti-proliferative properties of 8-PN, the effect of this compound on induction of apoptosis was investigated. One of the initial signs of apoptosis in cells is translocation of phosphatidylserine (PS) from the inner face of the plasma membrane to the outside of plasma membrane. Consequently, construction of the cells will be lost and disruption in cell cycle will occur. Once PS appears on the surface, fluorescent annexin-V will be conjugated to the PS [33]. This feature of the cells is used to measure the apoptosis of treated cancer cells. Apoptosis plays a remarkable role in regulating cell death and can be an important target in the treatment of numerous diseases, particularly cancer [34].

Our results demonstrate that 8-PN induces apoptosis through induction of extrinsic and intrinsic apoptotic pathways. In the presence of 8-PN caspase 8 and 9 which are the key factors for extrinsic and intrinsic apoptotic pathways were activated time dependently. These findings confirmed the Annexin results, which showed that HCT-116 cells pass through the early and late apoptotic pathways and the number of the dead cells was increased in the presence of 8-PN time dependently. Moreover, AO/PI staining shows some morphological changes in 8-PN treated cells, which considered as features of apoptosis.

The caspases cascade is groups of proteinases that have central roles in the activation of apoptosis. Activation of specific caspase determines the cell death pathways, for instance, activation of caspase-8 indicates the involvement of the extrinsic apoptosis pathway, while activation of caspase-9 suggests the intrinsic apoptosis pathway [35]. Our study however, indicates that 8-PN induces both extrinsic and intrinsic apoptosis pathways in HCT-116 cells through activation of caspase-8 and caspase-9, respectively. Previous study has been reported that 8-PN possess apoptotic properties against MCF-7 cells through activation of caspase-8, whereas intrinsic apoptosis pathway was not investigated [19].

Although in this study we had proven that 8-PN induces apoptosis in HCT-116 through intrinsic and extrinsic apoptosis pathways, further investigations are required to understand the exact mechanism by which 8-PN acts against HCT-116 at the protein and gene expression levels. Moreover, our data indicate that 8-PN inhibits cell proliferation

in G0/G1 phase, however the signaling pathways of cell cycle suppression is still unclear.

5. Conclusion

In conclusion, our results demonstrated that 8-PN induces apoptosis in HCT-116 colon cancer cells. This compound activates both the early and late stages of apoptosis as demonstrated via fluorescence microscopy and flow cytometry analysis. Furthermore, 8-PN may trigger both intrinsic and extrinsic apoptosis signaling pathways to cause cell death. In addition, 8-PN also induces cell cycle arrest at G0/G1 phase in HCT-116 cells in a time-dependent manner. Given the potential anticancer effect of 8-PN, further in-depth knowledge about the molecular mechanisms of cell death induced by 8-PN is urgently required for future research targeting colorectal cancer.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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