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Anti-oxidant, Anti-inflammatory, and Anti-pancreatic Cancer Activities of Cynaroside and n-butanol Fraction of *Orostachys japonicus*

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Abstract Cynaroside (luteolin-7-O-glucoside) is a flavone glycoside compound that has been reported to possess strong anti-oxidant activities. However, the integrative study for anti-inflammatory and anti-cancer activities of cynaroside alone or together with some flavonoids from medicinal herbs is rare. This study demonstrated anti-oxidant, anti-inflammatory, and anti-pancreatic cancer activities of cynaroside and n-butanol fraction derived from *Orostachys japonicus* (OJB). HPLC analysis indicated that OJB contained three different types of flavonoid glycosides; the major component among them was cynaroside. The anti-oxidant activities of cynaroside and OJB were validated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays and confirmed through analyzing the expression levels of heme oxygenase-1 (HO-1). The anti-inflammatory effects of them were certified by observing the decrease of lipopolysaccharide (LPS)-stimulated nitric oxide (NO) in RAW 264.7 cells. Additionally, the anti-proliferative activities of them were confirmed by 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS) assays in PANC-1 cells. Apoptotic bodies induced by them were clearly proven by DAPI staining. The cell cycle arrest at G1/S phase provoked by OJB was affirmed using flow cytometry. The effective activation of caspase -9, -8, -3 and inhibition of CDK2 and CDK4 by OJB were verified analyzing the protein levels measured using western blot. Such results indicated that both cynaroside and OJB effectively exerted anti-cancer effects on PANC-1 cells. In conclusion, the OJB containing cynaroside as a major flavonoid compound has the potential to be utilized as effectual anti-oxidant, anti-inflammatory, and anti-cancer agents in the future.

Keywords: *Orostachys japonicus, cynaroside, anti-oxidant, anti-inflammatory, anti-cancer*

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1. Introduction

Orostachys japonicus (*O. japonicus*), known as Wasong in Korea, is abundantly distributed in Korea and China. It has been reported that *O. japonicus* has been widely used as a folk medicine. *O. japonicus* contains bioactive phytochemicals, such as β-amyrin, friedelin, epi-friedelanol, glutinol, glutinone, campesterol, β-sitosterol, quercetin, kaempferol, and fatty acid esters [1]. Currently, most studies on *O. japonicus* have focused on flavonoids, which have potent anti-oxidant, anti-inflammatory, and anti-cancer properties [2-7]. However, there is no research about the anti-cancer activity of n-butanol fraction from *O. japonicus* in pancreatic cancer cells.

The n-butanol fraction from *O. japonicus* is composed of three substances such as cynaroside (luteolin-7-O-glucoside), kaemepferol-3,7-diglucoside, and kaempferol-3-rhamnosyl-7-glucoside. Among them, cynaroside is a major flavonoid of OJB with the molecular formula $C_{21}H_{20}O_{11}$. Anti-oxidant, anti-inflammatory, and anti-cancer activities of cynaroside have been demonstrated [8,9]. Thus, we expect that OJB will have anti-oxidant, anti-inflammatory, and anti-cancer effects.

Anti-oxidant compounds play an important role in protecting cells against free radicals and thus prevent the cells damage [10]. Free radicals, which are constantly generated in the metabolic process of living things, are molecular species with an unpaired electron in an atomic orbit [11]. Among these free radicals, reactive oxygen species (ROS) is one of the causes that induce oxidative stress, and lead to various damages to cellular tissues, inflammation, brain disease, heart disease, arterial disease, and cancer [12]. Normally, free radicals are destroyed by the body's natural anti-oxidant system, but sometimes the body's natural anti-oxidant system cannot cope with the free radicals [13]. Therefore, the study of anti-oxidants is an important issue to reduce oxidative stress and to inhibit the oxidation reaction of free radicals.

Hemeoxygenase-1 (HO-1), an important molecule of the anti-oxidant system, plays a crucial role in anti-oxidant properties [14]. In normal conditions, HO-1 expresses at a low level, but the expression level increases when oxidative stress is induced [15]. High expression level of HO-1, also, positively contributes to anti-inflammatory and anti-cancer activity [16].

The inflammation response is a physical or chemical defense mechanism against various stimuli such as infection, burn, and tissue injury [17]. Macrophage, which is an immune cell, induces inflammatory reactions by producing various types of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1beta (IL-1β) [18,19]. Activated macrophage cells produce inflammatory mediators like nitric oxide (NO) and prostaglandin E_2 (PGE_2) to promote inflammatory responses [20]. The production of NO is regulated by the inducible NO synthase (iNOS), and PGE_2 is elevated by the expression of cyclooxygenase-2 (COX-2) during the inflammation responses [21]. Therefore, NO and PGE_2 produced by iNOS and COX-2 may indicate the degree of inflammation [22]. Lipopolysaccharide (LPS)-induced iNOS and COX-2 expression increased the level of nuclear transcription factor Kappa-B (NF-κB). When NF-κB binds to inhibitory kappa B (IκB) in the cytoplasm, it exists in an inactivated state, and IκB is degraded by the proteasome1 [23]. Upregulated NF-κb translocates into the nucleus and induces transcription of iNOS and COX-2 [24,25]. Therefore, NF-κB is one of the important potential targets in the inflammatory system. When inflammation persists for a long time, it becomes chronic inflammation, which increases the risk carcinogenesis [26]. For these reasons, inhibition of oxidative stress and inflammation is necessary to prevent cancer.

Pancreatic cancer is the most deadly cancer and has a low survival rate, because early diagnosis of it is difficult [27]. Lifestyle factors such as smoking, alcohol consumption, obesity, and chronic pancreatitis increase the incidence of pancreatic cancer worldwide [28]. Despite improvements in treatments, new biomedical drugs and treatments are still necessary. Many studies have reported that the apoptosis-inducing effect *of O. japonicus* is effective against gastric [29], prostate [30], cervical [31], and pancreatic cancer [32].

Apoptosis (programmed cell death) plays an important role in physiological and pathological states. Defects in the apoptosis pathway can lead to cancers [33]. There are various conditions including DNA damage or uncontrolled proliferation in which the apoptotic pathway is activated [34]. In general, there are two apoptosis pathways. In the intrinsic pathway, cells are activated by cell damage, ischemia, and oxidative stress [35]. The phenomena lead to characteristic morphological changes which include cell blebbing and shrinkage [36]. In the extrinsic pathway the cell kills itself on the ground of signals from other cells. Both pathways cause a cells death through activating caspases that degrade other proteins. The apoptosome cleaves the pro-caspases-9 in the intrinsic pathway to active caspase-9, which in turn activates pro-caspase into the effector caspase-3. The activation of initiator pro-caspase-8 in the extrinsic pathway in turn activates effector caspase-3. Activated caspase-3 cleaves important cellular proteins such as poly (ADP-ribose) polymerase, ICAD-CAD, and lamins [37]. This activation of caspase-3 leads to DNA fragmentation and chromatin condensation. Weak external signals may also activate the intrinsic pathway of apoptosis.

The connection between the cell cycle and cancer is well known: cell cycle progression and arrest control cell division, and cancer is a result of abnormal cell proliferation. Normal cells keep a typical cell cycle, grow, divide, and die. Cancer cells, on the other hand, multiply and continue to reproduce abnormal cells instead of dying. There are four stages to the cell cycle: G1 phase, S phase, G2 phase, and M phase. There are also three checkpoints; the G1/S checkpoint; the G2/M checkpoint; and the metaphase checkpoint [38]. These checkpoints tell the overall condition when the process passes to the next cell cycle. CDKs are crucial modulators for cell cycle progression and arrest. When cyclin binds to CDK, CDK becomes activated and induces the cell cycle progression [39].

Thus, the aim of the current study is to investigate the anti-oxidant, anti-inflammatory, and anti-cancer activities of cynaroside and n-butanol fraction of *O. japonicus* using RAW 264.7 mouse macrophage cells and PANC-1 human pancreatic cancer cells.

2. Materials and Methods

2.1. Cell Lines and Reagents

PANC-1 human pancreatic cancer cells and murine RAW 264.7 macrophage cells were bought from the Korea cell line bank (KCLB, Seoul, Korea). RPMI-1640 medium, Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), streptomycin, and penicillin were supplied from Hyclone (Logan, UT). Cynaroside was bought from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Monoclonal antibodies against HO-1, PGE_2, COX-2, iNOS, NF-κB, IκB, caspase-3, caspase-8, caspase-9, cleaved caspase-8, cleaved caspase-9, CDK2, CDK4, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were supplied from Cell Signaling Technology Inc. (OR, USA). The secondary antibody was, also, bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All others used were of the highest grade available.

2.2. HPLC Analysis

HPLC analysis was conducted using a liquid chromatography system of Agilent 1100 series (Palo Alto, CA, USA), which includes a vacuum degasser, quaternary gradient pump, autosampler, and diode array detector (DAD). It was connected to Agilent ChemStation software. A Zorbax octadecylsilane (ODS) C18 column (250mm x 4.6mm id, 5μm) from Phenomenex (Torrance, CA) was used at 25°C. The mobile phase contained deionized water (solvent A) and 1% formic acid (solvent B), and UV detection was operated at 350nm at a flow rate of 1 mL/min. Each of 10 μL of cynaroside and OJB was injected at a flow rate of 1 mL/min.

2.3. Cell Culture

At 37°C and 5% CO_2, PANC-1 cells were harvested in DMEM with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cancer cells were subcultured every week at 1:3 split ratios using trypsin, and every 3 days, medium was changed. Cells were cultured for 24 h (approximately 80 to 90% confluency). Cells were treated with the vehicle (0.1% DMSO), cynaroside, and various concentrations of *O. japonicus*. RAW 264.7 cells were cultured at 37°C and 5% CO_2 in RPMI 1640 containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. RAW 264.7 cells were subcultured every week at a 1:3 ratio using a cell scraper, and the medium was changed every 3 days. Cells were harvested after 24 h (approximately 90% confluency). RAW 264.7 cells were stimulated with 10 μg/mL LPS and then treated with the vehicle (0.1% DMSO) and various concentrations of cynaroside and OJB.

2.4. DPPH Radical Scavenging Assay

For the measurement of anti-oxidant activity, cynaroside and OJB were dissolved in distilled water. Each samples were diluted to various concentrations (cynaroside was diluted to 25, 50, 75, 100, 200 μM and OJB diluted to 25, 50, 75, 100, 200 μg/mL). The reaction mixture containing 0.5 mL of each sample and 0.5 mL of the DPPH radical solution was left for 30 min at 25°C in the dark. The absorbance was measured by an immunosorbent assay (DI BIOTEK, Synergy HT) at 517 nm. Using the formula below, the percentage of scavenging activity was determined.

$$Scavenging\ activity\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

2.5. Cytotoxicity Assay

MTS assay was used to confirm the cell toxicity of OJB and cynaroside. In the cell toxicity assessment, RAW 264.7 cells were seeded in a 96-well plate with RPMI media. A serum-free medium (SFM) without LPS or SFM with LPS (10 μg/mL) was, then, applied and incubated at 37°C and 5% CO_2 for 12 h. Subsequently, OJB (25, 50, 75, and 100 μg/mL) and cynaroside (100 μM) extracts were added to RAW 264.7 cells and cultured for 24 h. After cultured, the medium was removed and MTS solution was added. The 96-well plate was incubated for 3 h in the dark and then absorbance was measured at 490 nm using a microplate reader (PowerWaveXS, BioTek, VT, USA). This test was repeated three times and cell viability was calculated.

2.6. NO Assay

In a 96-well plate, RAW 264.7 cells were cultivated at a density of 5×10^5 cells/mL for 24 h, and then kept alive in serum-free medium (SFM) with or without LPS for 12 h. Cells were treated with final concentrations of OJB (25, 50, 75, 100 μg/mL) and cynaroside (100 μM). The NO inhibition activity was analyzed by Griess assay. The Griess reagent (0.1% naphthylethylenediamnine and 1% sulfanilamide in 5% H_3PO_4 solution) (Sigma) was mixed to each of the supernatant from the cells treated with samples. NO contents were then read at 540nm.

2.7. Cell Viability Assay

A CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI. USA) was used to measure cell viability. PANC-1 cells were maintained in serum-free media (SFM) for 12 h after being seeded onto 96-well plates at a density of 5.0×10^5 cells/mL and incubated at 37°C for 24 h. PANC-1 cells were incubated with various concentrations of OJB (3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) and cynaroside (10, 20, 40, 80, 100, and 200 μM) for 24 h. MTS solution was added after incubation, and the cells were subsequently grown for 3 h at 37°C in a humid environment with 5% CO_2. A microplate reader was then used to measure absorbance at 490 nm. This assay was performed at least three times.

2.8. 4,6-Diamidino-2-Phenylindole (DAPI) Assay

PANC-1 cells were treated with OJB and cynaroside. The cells were washed with PBS before being fixed in a 4% paraformaldehyde reagent at room temperature for 20 min. Fixed cells were washed with PBS, and then the coverslips were mounted with DAPI (Vector Laboratories, CA, USA) solution. The cell nuclei were observed using confocal microscopy of Carl Zeiss LSM 800.

2.9. Cell Cycle Analysis

Using a Cell Cycle Phase Determination Kit (Cayman Chemical, Ann Arbor, MI, USA), DNA fragments were stained with PI to determine the cell cycle phase. PANC-1 cells were first cultured in a 6 well plate (5×10^5 cells/mL), and then cultured in SFM medium for 12 h. After being exposed to various OJB concentrations for 12 h, the cells were collected. The harvested cells were centrifuged at 10,000 × g, twice washed in an assay buffer, and then resuspended at a density of 4×10^5 cells/mL. 1mL of a fixative was added to the cells, and they were then permeabilized and fixed for over 2 h. Following treatment, fixatives were decanted, cell pellets were suspended in 500 μL of a staining solution containing 200 μL of DNase-free RNase and a 200 μL of PI, and incubated at room temperature for 30 min in the dark. Using flow cytometry with the FACSCalibur, the cells were examined.

2.10. Western Blot Analysis

The cells were then centrifuged into pellets, the pellets were resuspended in lysis buffer and kept on ice for 1 h, and the cell debris was spun at 10,000 × g for 10 min to remove it. BCA protein assay was used to determine protein concentrations (Pierce, Rockford, IL, USA). For electrophoresis, 40 μg of protein samples were separated on polyacrylamide gels using 10-15% SDS-PAGE (Bio-Rad, CA, USA). The gel was transferred onto a PVDF membrane using a semidry transfer method after

electrophoresis (Bio-Rad, CA, USA). The membrane was blocked for 2 h using PBST solution (PBS with 0.1% Tween 20) before overnight primary antibody incubation. The membrane was cleaned with PBST three times, and then incubated with secondary antibody for 2 h at room temperature and lastly cleaned with PBST three times. Using ECL detection kits, the final detection was performed (Santa Cruz, CA, USA).

3. Results

3.1. HPLC Analysis of OJB

By comparing the retention times of OJB (100 μg/mL) and cynaroside (1000 μM) in the chromatograms, we found that the observed major component at 350 nm was identified as cynaroside. As shown in Figure 1, cynaroside (B) peak was seen at 9.740 min of retention time, which was almost in accord with the peak at 9.956 min of retention time of OJB.

3.2. Effects of Cytotoxicity of OJB and Cynaroside on RAW 264.7 Cells

To determine the cytotoxicity effects of OJB and cynaroside on RAW 264.7 macrophage cells, the MTS assay was used with and without LPS pretreated. In RAW 264.7 cells without LPS, 100 μg/mL of OJB was applied for 24 h. In order to examine the effect of LPS on RAW 264.7 cells, OJB (25, 50, 75, and 100 μg/mL) and cynaroside (100 μM) were added onto LPS-induced RAW 264.7 cells for 12 h. As a result, OJB and cynaroside showed no cytotoxicity on RAW 264.7 cells (Figure 2).

3.3. DPPH Radical Scavenging Activity of OJB and Cynaroside

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay was used to evaluate the free radical scavenging activity of OJB and cynaroside. Both OJB (25, 50, 75, and 100 μg/mL) and cynaroside (25, 50, 75, and 100 μM) showed increasing DPPH radical scavenging activities in a dose-dependent manner. It was confirmed that the DPPH radical scavenging rate of OJB was almost similar to that of cynaroside (Figure 3).

3.4. The Expression Level of HO-1 Influenced by OJB and Cynaroside in LPS-induced RAW 264.7 Cells

The HO-1 signaling pathway is an anti-oxidant system that inhibits oxidative stress. To investigate the anti-oxidant effect caused by the increase in HO-1 protein level, the effects of OJB and cynaroside were studied by western blot. Results showed that HO-1 protein levels increased in a concentration-dependent manner in RAW 264.7 cells induced with LPS and then treated with OJB (25, 50, 75, and 100 μg/mL) and cynaorside (100 μM) for 12 h (Figure 4). This data represents that the anti-oxidant effect of both OJB and cynaroside is associated with the HO-1 signaling pathway.

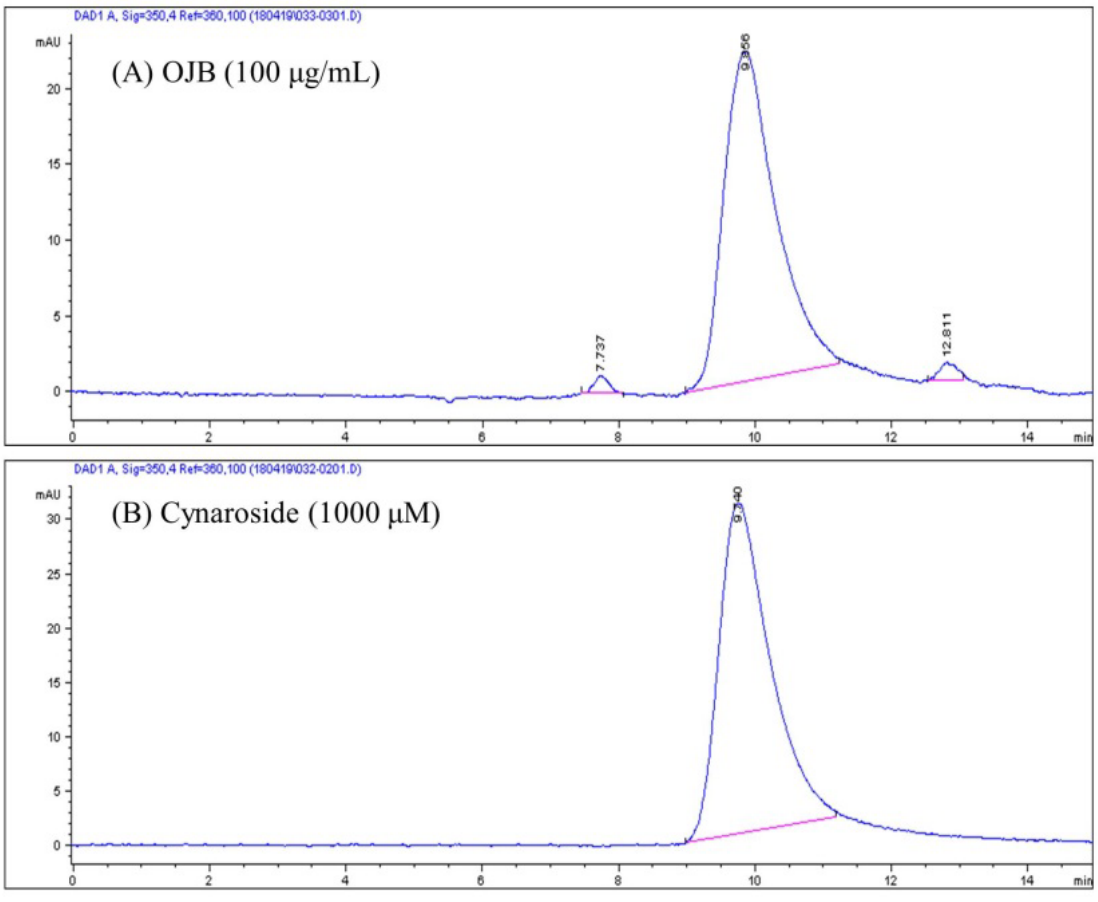

Figure 1. Chromatograms of n-butanol fraction from *Orostachys japonicus* (100 μg/mL) (A) and of cynaroside (1000 μM) (B)

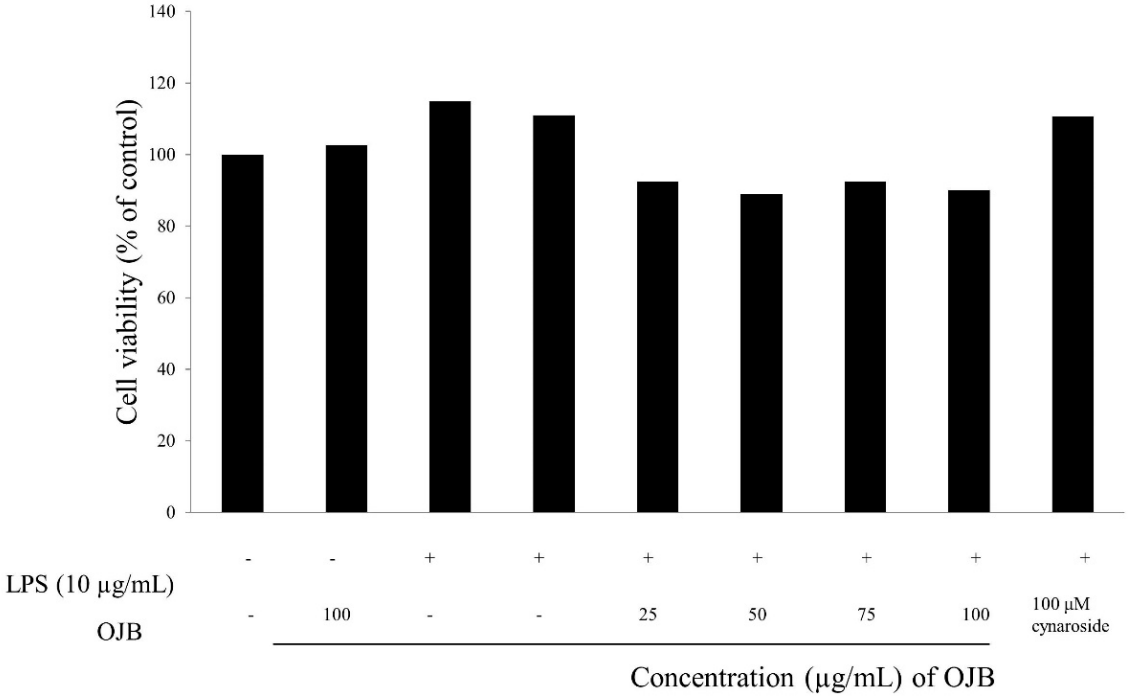

Figure 2. Effects of OJB and cynaroside on viabilities of RAW 264.7 cells. The cell viabilities were determined using MTS assay. RAW 264.7 cells were treated with OJB (100 μg/mL) for 12 h. LPS-indcued RAW 264.7 cells were treated with various concentrations of OJB (25, 50, 75, and 100 μg/mL) and cynaroside (100 μM) for 12 h. The mean ± standard deviation are used to express data (n = 3)

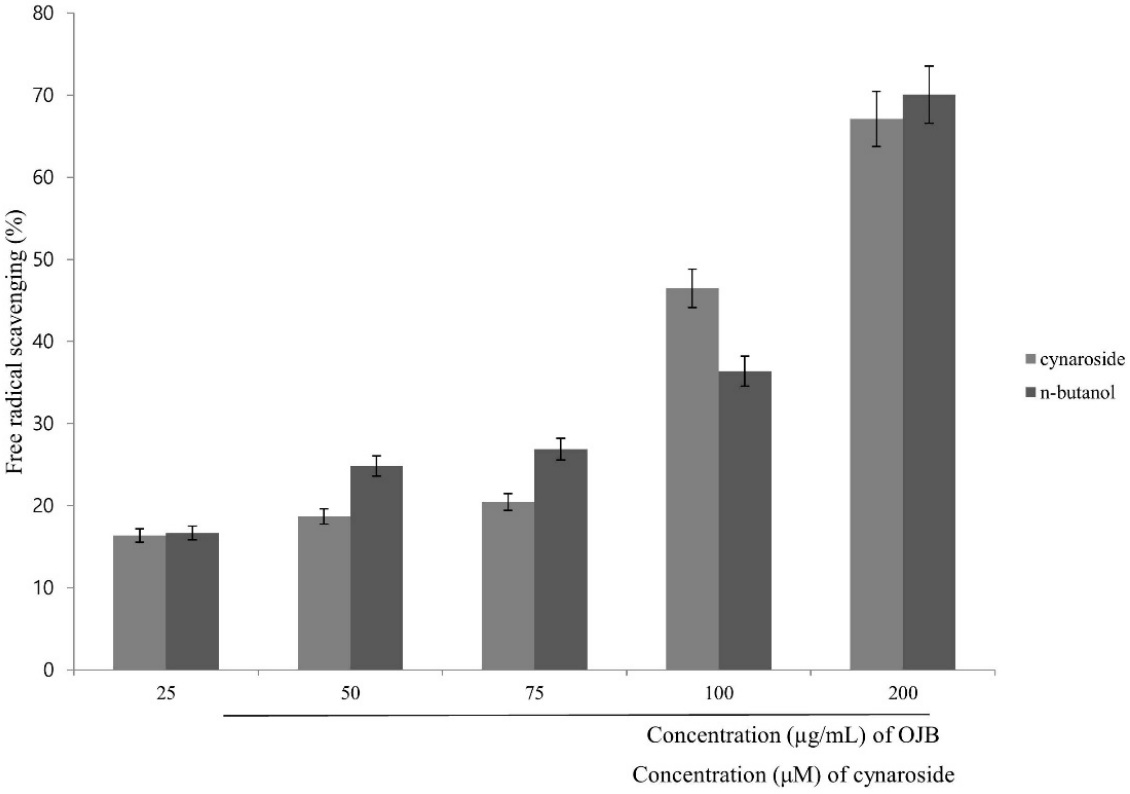

Figure 3. Effects of cynaroside and OJB on DPPH radical scavenging activity. Bars represent arithmetic mean ± SD (n=3)

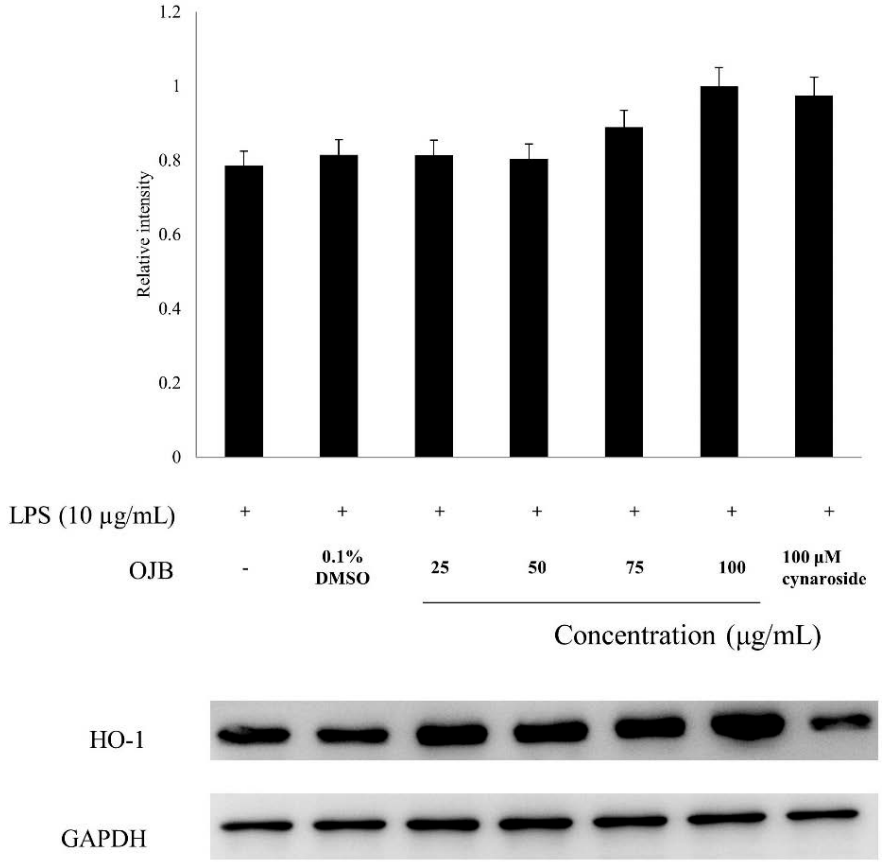

Figure 4. Effects of OJB and cynaroside on the expression levels of HO-1. Cells were incubated with OJB (25, 50, 100 μg/mL) and cynaroside (100μM) in the presence of LPS (10 μg/mL). Western blot analysis was utilized to measure the expression levels of HO-1 of cell lysates (15 μL). The density of bands was quantitated. The internal control was GAPDH. Similar results were obtained in three other tests in which band intensities were measured using a densitometry

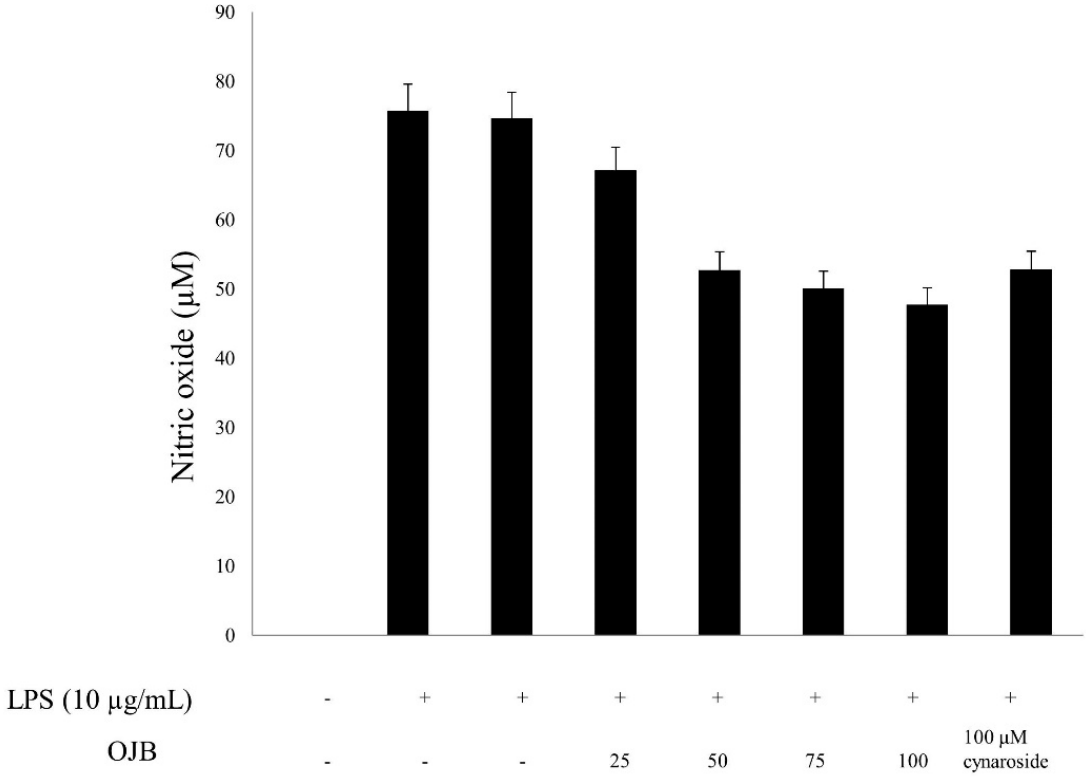

Figure 5. Effects of OJB and cynaroside on the production levels of nitric oxide. Cells were incubated with OJB (25, 50, 75, and 100 μg/mL) and cynaroside (100 μM) in the presence of LPS (10 μg/mL). NO productions in culture supernatants were measured using the Griess method. Data represent the mean ± SD

3.5. Inhibition of NO Formation by OJB and Cynaroside

To confirm the anti-inflammatory activity of OJB and cynaroside, the formation of NO in a concentration-dependent manner was measured in RAW 264.7 cells treated with and without LPS. NO levels were significantly increased in RAW 264.7 cells treated with LPS for 12 h. OJB (25, 50, 75, and 100 μg/mL) and cynaorside (100 μM) were shown to significantly reduce NO production (Figure 5). These results indicate that OJB and cynaroside inhibit the production of NO, an proinflammatory mediator, in RAW 264.7 cells pretreated with LPS.

3.6. The Expression Level of iNOS Influenced by OJB and Cynaroside in LPS-induced RAW 264.7 Cells

To confirm that the decrease in iNOS protein level was the cause of the decrease in NO production, the effects of OJB and cynaroside treated on RAW 264.7 cells were studied by western blot. As a result of treating LPS-induced RAW 264.7 cells with OJB (25, 50, 75, and 100 μg/mL) and cynaorside (100 μM) for 12 h, iNOS protein levels were suppressed (Figure 6). Consequently, it can be seen that the reduction of iNOS expression by OJB and cynaroside is the cause of the inhibition of NO production.

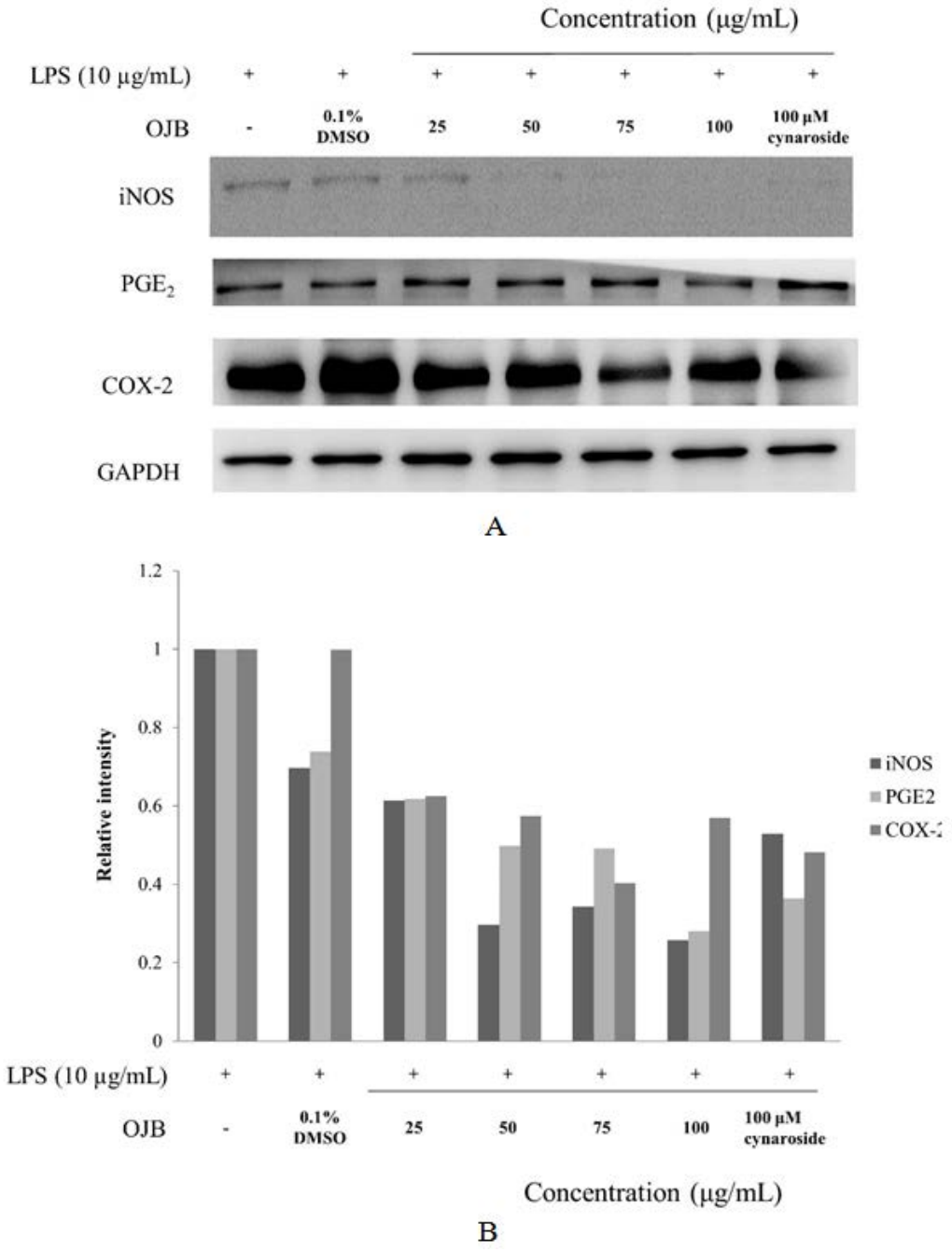

Figure 6. Effects of OJB and cynaroside on the production levels of iNOS, PGE_2, and COX-2. Cells were incubated with OJB (25, 50, 75, and 100 μg/mL) and cynaroside (100μM) in the presence of LPS (10 μg/mL). Cell lysates (15 μL) were used to measure the production levels of iNOS, PGE_2, and COX-2 by western blot. The density of bands was quantitated. The internal control was GAPDH. Similar results were obtained in three other tests in which band intensities were measured using a densitometry. The intensity of band (A) was quantified and represented as the bar graph (B)

3.7. The Production Level of PGE_2 Influenced by OJB and Cynaroside in LPS-induced RAW 264.7 Cells

PGE_2 is a pro-inflammatory mediator in the inflammatory response. The production level of PGE_2 by western blot. was measured. When RAW 264.7 cells, induced with LPS for 12 h, were treated with OJB (25, 50, 75, and 100 μg/mL) and cynaorside (100 μM) for 24 h, it was confirmed that PGE_2 production was inhibited (Figure 6). These results indicate that treatment with OJB and cynaroside suppresses the production of the proinflammatory mediator PGE_2.

3.8. The Expression Level of COX-2 Influenced by OJB and Cynaroside in LPS-induced RAW 264.7 Cells

When RAW264.7 cells, induced by LPS for 12 h, were treated with OJB (25, 50, 75, and 100 μg/mL) and cynaorside (100 μM) for 24 h, COX-2 expression level was suppressed in these cells (Figure 6). Consequently, the reduction of COX-2 expression by OJB and cynaroside causes the reduction of PGE_2 production.

3.9. The Expression Level of NF-κB Influenced by OJB and Cynaroside in LPS-induced RAW 264.7 Cells

NF-κB is a transcription factor that regulates iNOS and COX-2. We observed whether OJB and cynaroside prevent the translocation of NF-κB to the nucleus by Western blot. When RAW 264.7 cells, pretreated with LPS for 12 h, were treated with OJB (25, 50, 75, and 100 μg/mL) and cynaorside (100 μM) for 24 h, it was confirmed that the level of NF-κB protein in these cells was reduced (Figure 7). Additionally, we investigated whether OJB and cynaroside block the degradation of IκB by western blot. IκB protein levels were increased in RAW 264.7 cells treated with OJB (25, 50, 75, and 100 μg/mL) and cynaorside (100 μM) (Figure 7). The results indicate that the anti-inflammatory effects of OJB and cynaroside in LPS-induced RAW 264.7 cells follow the NF-κB pathway.

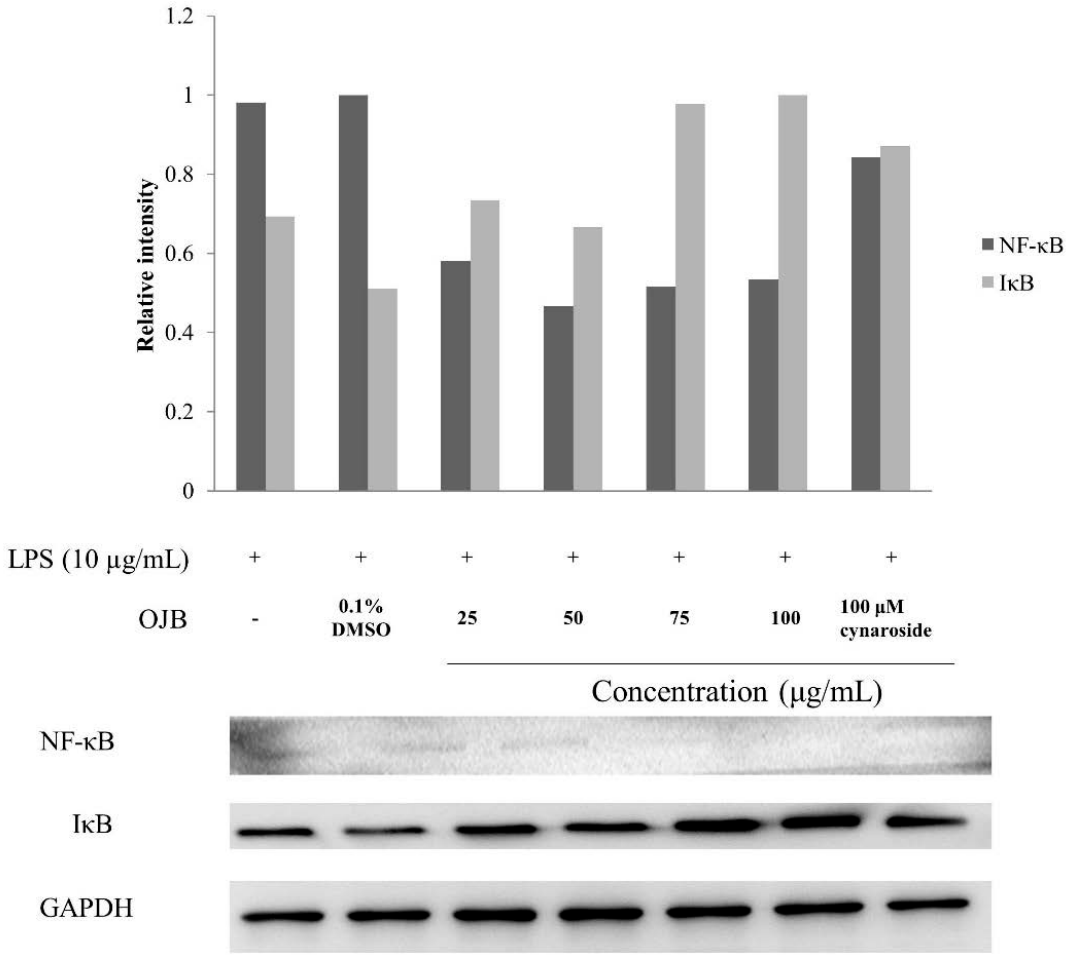

Figure 7. Effects of OJB and cynaroside on the production levels of NF-κB and IκB. Cells were incubated with OJB (25, 50, 75, and 100 μg/mL) and cynaroside (100μM) in the presence of LPS (10 μg/mL). Cell lysates (15 μL) were used to measure the production levels of NF-κB and IκB by western blot. The density of bands was quantitated. The internal control was GAPDH. Similar results were obtained in three other tests in which band intensities were measured using a densitometry.

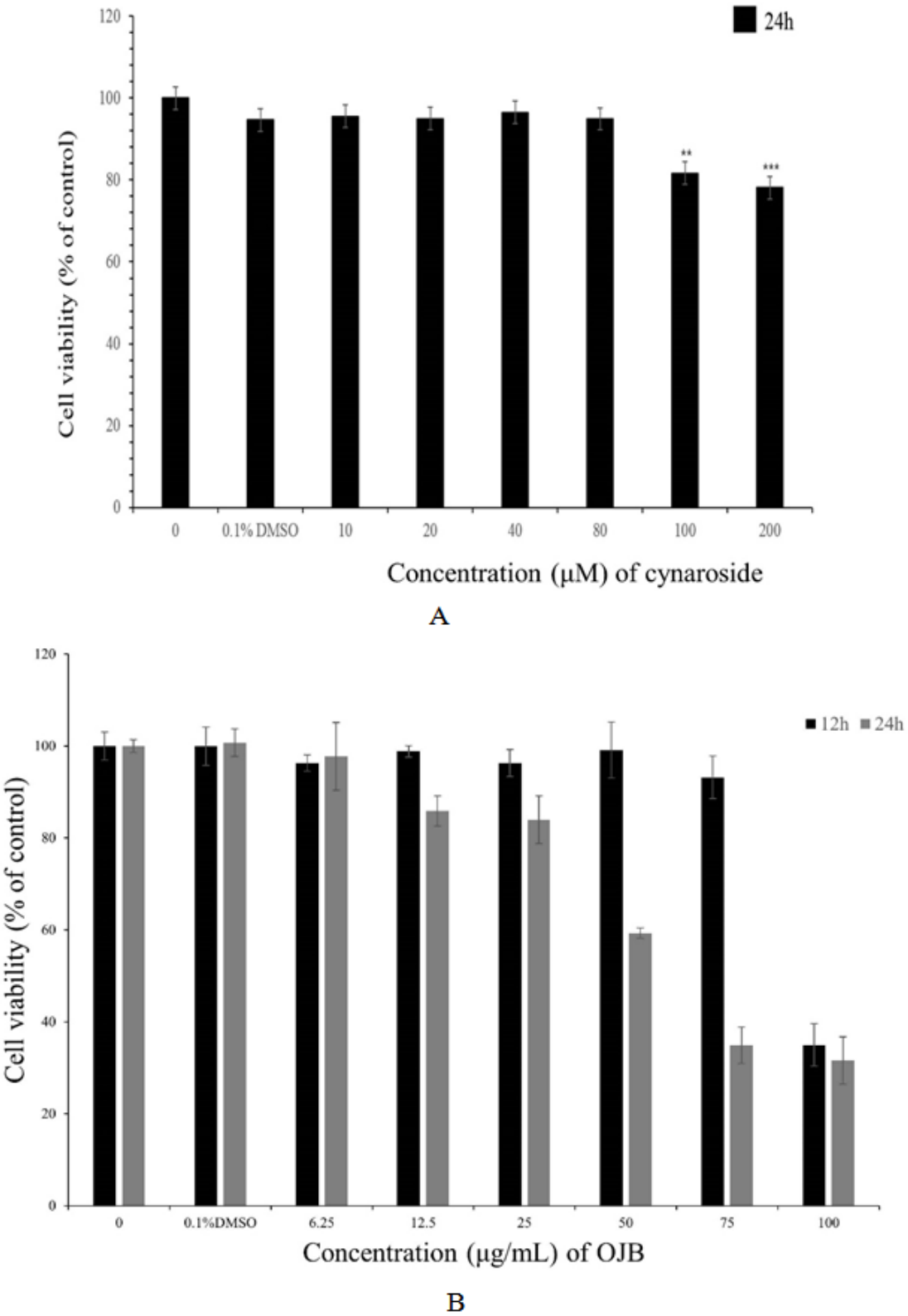

Figure 8. Effects of cynaroside and OJB on viabilities of PANC-1 cells. The cell viabilities were determined using MTS assay. PANC-1 cells were treated with 0.1% DMSO and different concentrations of (A) cynaroside (10, 20, 40, 80, 100, 200 μM) for 24 h and (B) OJB (6.25, 12.5, 25, 50, 75, 100 μg/mL) for 12 and 24 h. The mean ± standard deviation is used to express data (n = 3). *$p < 0.05$, **$p < 0.001$ versus control

3.10. Inhibition of Proliferation of PANC-1 Cells by OJB and Cynaroside

To confirm the effects of OJB and cynaroside on the proliferation of pancreatic cancer cell line PANC-1 human pancreatic cancer cells, MTS analysis was performed. PANC-1 cells were treated with cynaroside (10, 20, 40, 80, 100, and 200 μM) for 12 h and OJB (6.25, 12.5, 25, 50, 75, and 100 μg/mL) for 12 and 24 h. OJB significantly inhibited the proliferation of PANC-1 cells in a dose- and time-dependent manner (Figure 8A). On the other hand, cynaorisde had a lower inhibitory effect on PANC-1 cells than OJB (Figure 8B). These results represent that OJB effectively inhibits the growth of pancreatic cancer cells.

3.11. Induction of Apoptosis of PANC-1 Cells by OJB and Cynaroside

The formation of apoptotic bodies by programmed cell death was evaluated by DAPI staining. PANC-1 human pancreatic cancer cells were treated with OJB and cynaroside for 24 h and stained with DAPI to examine morphological changes in the nucleus. DAPI staining results showed that OJB (25, 50, and 75 µg/mL) and cynaroside (100 µM) induced apoptosis of PANC-1 cells in a concentration-dependent manner, resulting in increased condensed chromatin and fragmented nuclei in these cells (Figure 9).

3.12. Effects of OJB on Expression of Apoptosis Associated Caspases in PANC-1 Cells

Activated caspases are closely related to apoptosis. Therefore OJB were checked whether activated caspases involved in the intrinsic and extrinsic pathways in PANC-1 cells. Western blotting studies showed that OJB activates caspase-9, which is an initiator in the intrinsic pathway, caspase-8, which is involved in the extrinsic pathway, and caspase-3, which is an executioner caspase interacting with caspase-9 and caspase-8 in both apoptosis pathways, in a concentration-dependent manner in PANC-1 cells (Figure 10).

3.13. Effects of OJB on Expression of Cell Cycle Arrest Associated CDKs in PANC-1 Cells

Using flow cytometry, the cell cycle progression of PANC-1 cells was examined. As shown in Figure 11, the sub-G1 phase of the cell cycle increased from 64.46% in control cells to 90.38 % in cells treated with 50 µg/mL of OJB. In PANC-1 cells treated with OJB, the cell population in the sub-G1 phase was increased in a concentration-dependent manner. In PANC-1 cells treated with OJB, cells in G1 phase were, also, decreased in a concentration-dependent manner compared to control cells (Table 1). Western blotting analysis, also, showed that OJB effectively induces cell cycle arrest by down-regulating the protein levels of CDK2 and CDK4, which are involved in the G1/S checkpoint, in a concentration-dependent manner in PANC-1 cells (Figure 12).

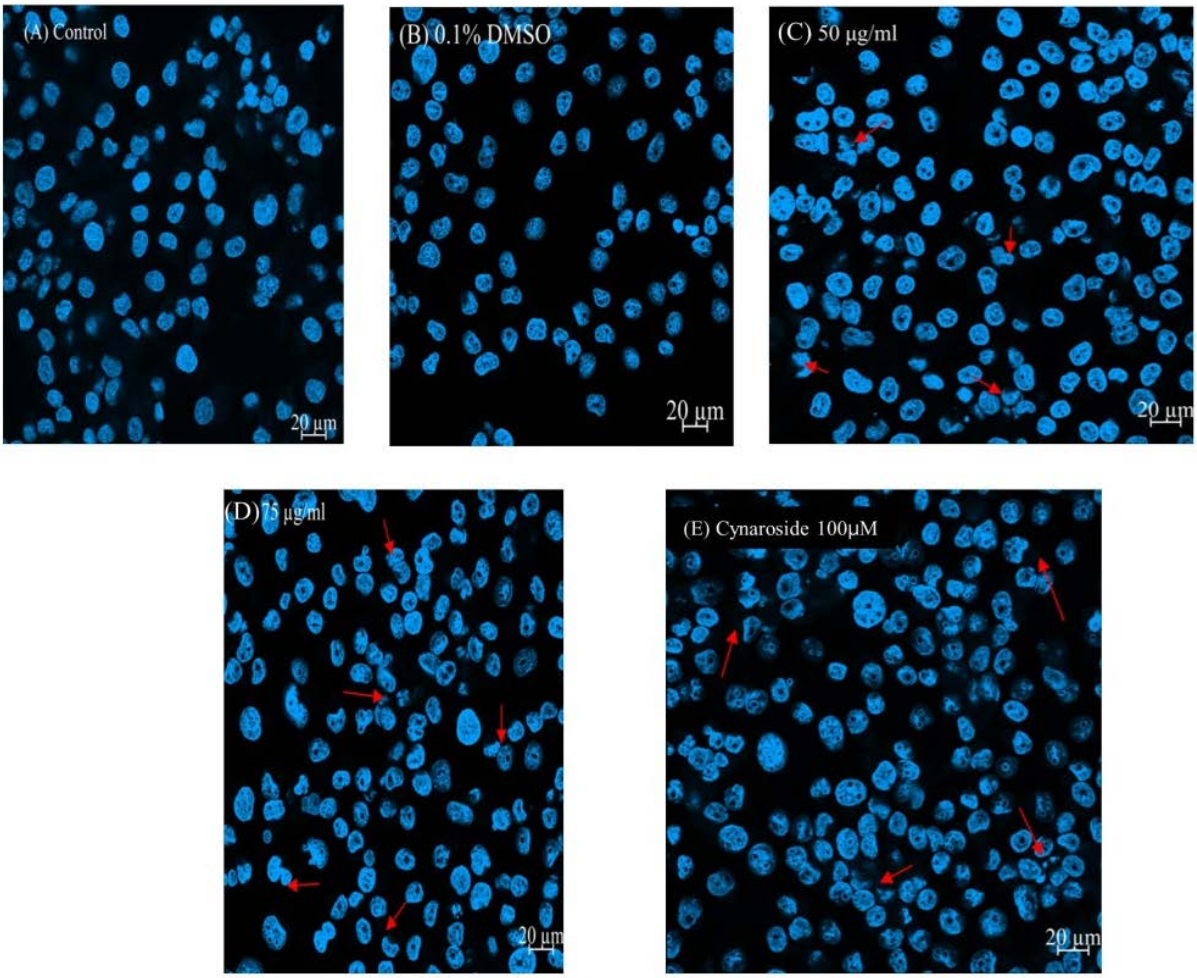

Figure 9. DAPI staining of apoptotic bodies of PANC-1 cells. Cells were treated for 12 h with the control (A), 0.1% DMSO (B), 50 (C), 75 (D) µg/mL of OJB and 100 (E) µM cynaroside. After being labeled with DAPI, cells were analyzed under confocal microscopy to look for morphological traits linked to apoptosis. Red arrows signify morphological changes

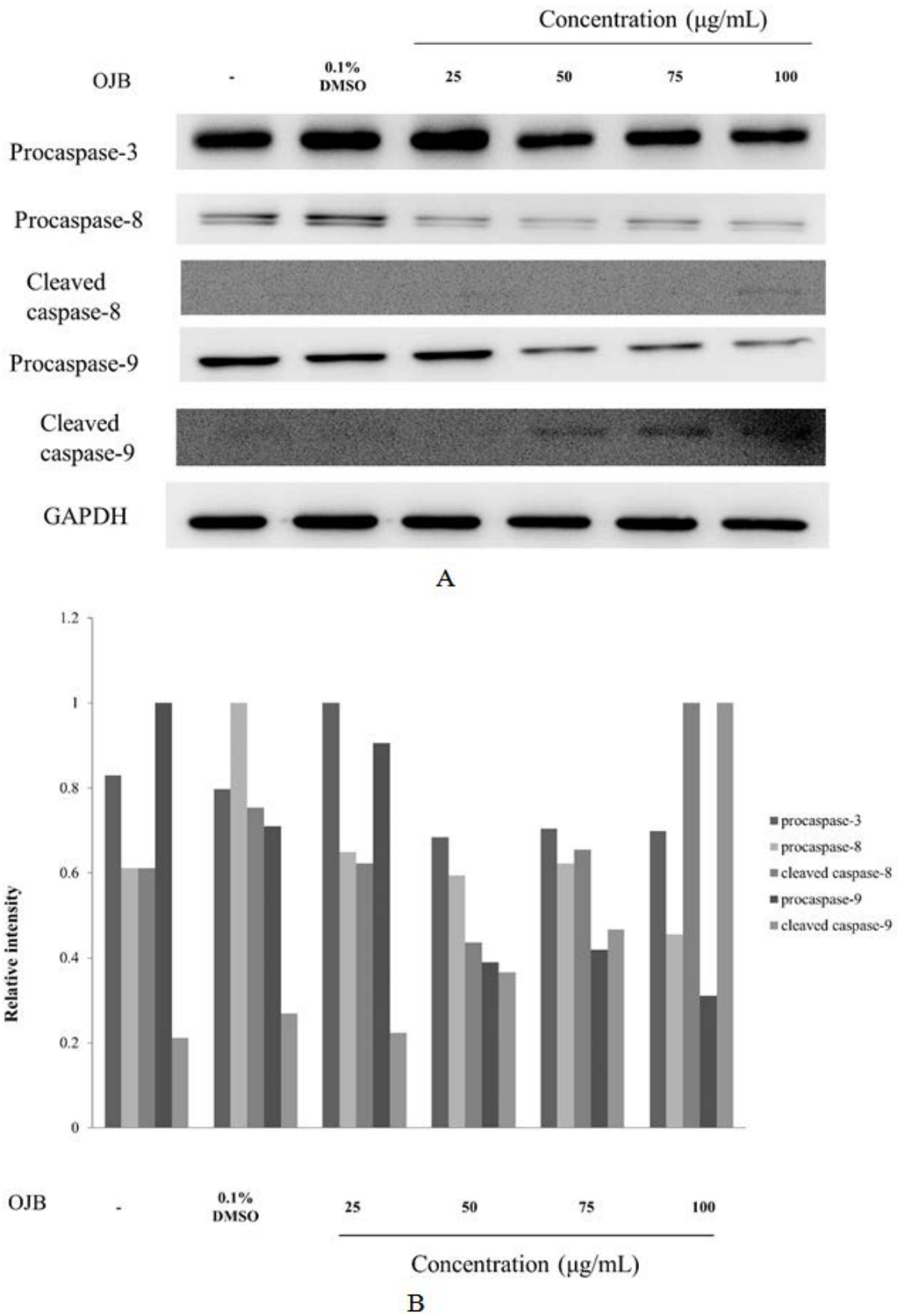

Figure 10. Effects of OJB on the protein levels of procaspase-3, -8, -9 and cleaved caspase-8, -9. OJB (control, 0.1% DMSO, 25, 50, 75, or 100 μg/mL) was applied to the cells for 12 h. The protein levels of procaspase-3, -8, -9 and cleaved caspase-8, -9 were examined by western blot. Band density was quantitated. The internal control was GAPDH. Similar results were obtained in three other tests in which band intensities were measured using a densitometry. The intensity of band (A) was quantified and represented as the bar graph (B)

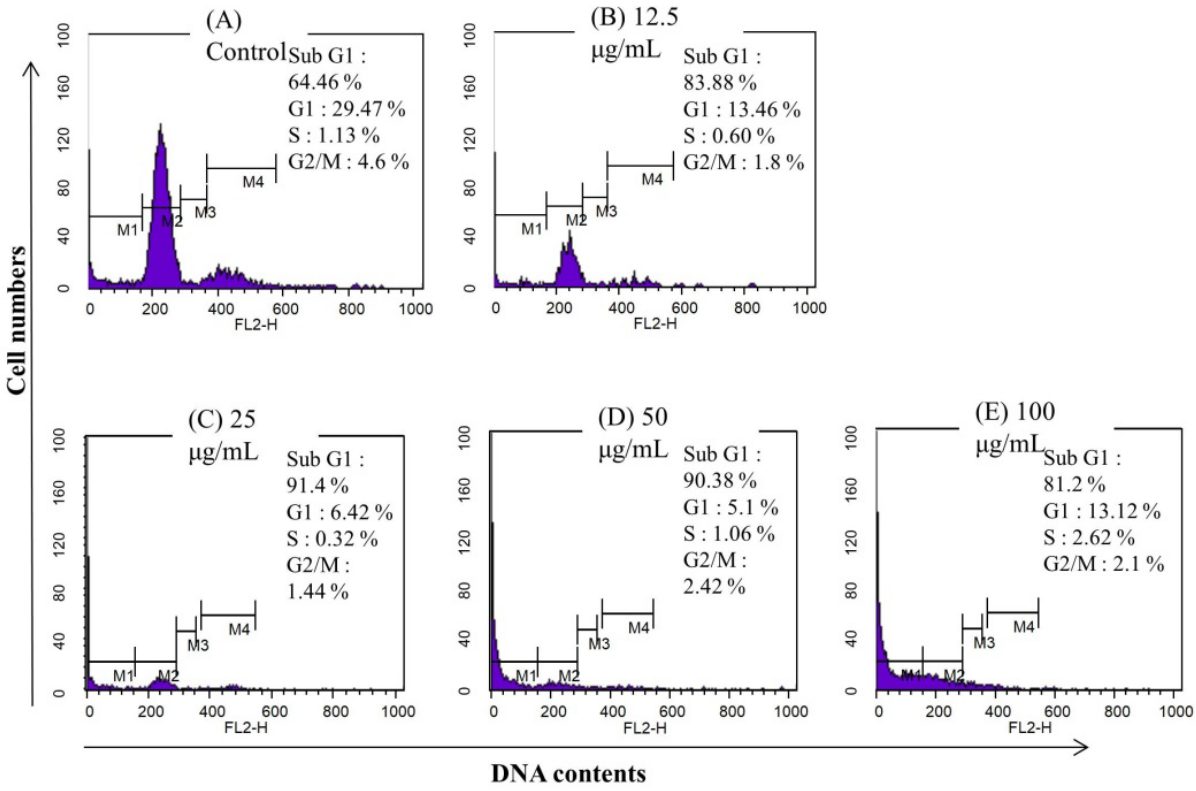

Figure 11. Flow cytometry analysis of cell cycle distribution of PANC-1 cells. Cells were treated with control (A), 0.1% DMSO (B), 25 (C), 50 (D), and 100 (E) µg/mL of OJB for 12 h. Histograms of PANC-1 cells show the sub-G1, G1, S, and G2/M phases

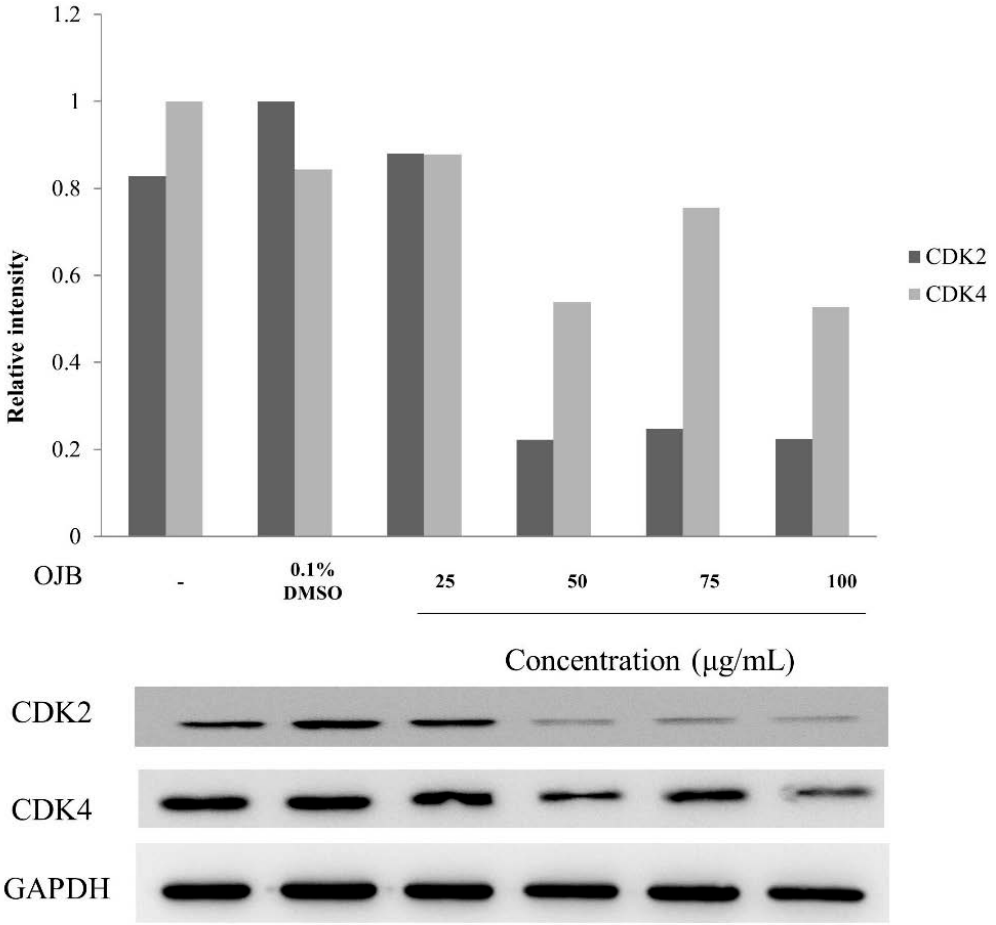

Figure 12. Effects of OJB on the protein levels of CDK2 and CDK4. OJB (control, 0.1% DMSO, 25, 50, 75, or 100 µg/mL) was used to treat the cells for 12 h. The protein levels of CDK2 and CDK4 were examined by western blot. The density of bands was quantitated. The internal control was GAPDH. Similar results were obtained in three other tests in which band intensities were measured using a densitometry

Table 1. Analysis of cell cycle distribution (%) using flow cytometry in PANC-1 cells treated with various concentrations of OJB

Concentration of OJB (μg/mL)	Sub G1 (M1)	G1 (M2)	S (M3)	G2/M (M4)
Control	64.46	29.47	1.13	4.6
12.5	83.88	13.46	0.60	1.8
25	91.4	6.42	0.32	1.44
50	90.38	5.1	1.06	2.42
100	81.2	13.12	2.62	2.1

4. Discussion

Various cancer treatments and anti-cancer drugs have been developed and used, but the exact method for preventing cancer is still not well known. Therefore, many people still rely on herbs and plants to fight cancer [40]. Because natural bioproducts have fewer side effects and toxicity, they have been developed as health products and medicines with anti-oxidant, anti-inflammatory, and anti-cancer activities [41,42]. *O. japonicus* is a medical herb that is used as a folk remedy for various treatments. In the present study, the *O. japonicus* n-butanol extracts were evaluated whether not only, inhibits oxidative stress and inflammation, but also, has high anti-pancreatic cancer activity. The first step in cancer prevention is to inhibit oxidative stress and chronic inflammation. Thus, in the current study, its anti-oxidant, anti-inflammatory, and anti-pancreatic cancer properties were analyzed.

The reason for using the butanol fraction of *O. japonicus* is that OJB contains cynaroside, a flavonoid with potential anti-oxidant, anti-inflammatory, and anti-cancer activities, which was confirmed by HPLC. In previous papers, there were no data systematically comparing OJB and cynaroside in terms of anti-oxidant, anti-inflammatory, and anti-cancer activities. In most of the comparison results, the effect of OJB was better, but since OJB and cynaroside had to be used at different concentrations, it cannot be asserted that OJB is more effective than cynaroside. However, such study revealed that the effects of OJB were expressed through cynaroside.

First, the anti-oxidant effects of OJB and cynaroside were verified by confirming DPPH scavenging activity and inducing HO-1 in RAW 264.7 cells. As a result, both OJB and cynaroside exhibited DPPH free radical scavenging activity in a concentration-dependent manner (Figure 3). In addition, when treated with OJB and cynaroside, respectively, the expression level of HO-1, which acts as a major enzyme for anti-oxidant activity, increased in a concentration-dependent manner in RAW 264.7 cells (Figure 4).

The anti-inflammatory effects of OJB and cynaroside were investigated using LPS-pretreated RAW 264.7 cells. The anti-inflammatory effects of OJB and cynaroside were found to suppress the production of NO and PGE_2, which are inflammatory cytokines induced by LPS, and this was found to be due to inhibition of the NF-κB signal pathway. iNOS and COX-2 are key enzymes that produce NO and PGE_2, respectively, and the production of these enzymes is regulated by the NF-κB signaling pathway [43,44]. OJB and cynaroside down-regulate iNOS and COX-2 protein levels in a concentration-dependent manner was confirmed by the western blot assay (Figure 5, Figure 6). In addition, in LPS-induced RAW 264.7 cells, OJB and cynaroside inhibited NF- κB nuclear translocation and IκB degradation (Figure 7) [45]. Based on these results, OJB and cynaroside can be used for the treatment of chronic inflammatory disease was evaluated.

Finally, the mechanisms of apoptosis and cell cycle arrest induced by OJB in PANC-1 pancreatic cancer cells were established. MTS assay results showed that cynaroside and OJB suppressed the viability of PANC-1 cells in a concentration-dependent manner (Figure 8). Apoptosis, one of the major mechanisms to inhibit cancer cell growth, has been an effective approach for most anti-cancer drugs. Morphological hallmarks of apoptosis include chromatin condensation, membrane blebbing, cell shrinkage, and formation of apoptotic bodies. These morphological changes in cells can be suggestive of apoptosis and provide a reliable basis for identifying cells undergoing apoptosis. DAPI Staining showed nuclear fragmentation, chromatin condensation, and apoptotic bodies in OJB or cynaroside treated PANC-1 cells (Figure 9). Two different pathways are known for apoptosis, one is the intrinsic pathway (mitochondria-dependent pathway) and the other is extrinsic pathway (death receptor-dependent pathway), which is divided according to whether the initiating signal originates inside or outside the cell [46,47,48]. In the present study, PANC-1 cells treated with OJB increased the levels of cleaved caspase-9 and -8 and decreased the expression of procasepase-9, -8, and -3 in a concentration dependent manner. In PANC-1 cells, a decrease in procaspase-9 and an increase in cleaved caspase-9 indicate intrinsic apoptosis, whereas a decrease in procaspase-8 and an increase in cleaved caspase-8 indicate extrinsic apoptosis. In addition, the decrease in procaspase-3 indicates the activation of caspase-3, which is responsible for executive functions in both intrinsic and extrinsic apoptosis, suggesting that OJB induces both pathways of apoptosis (Figure 10). Since cancer is an abnormal cell growth and proliferation phenomenon, cell cycle arrest is another excellent sign of anti-cancer activity [36]. To observe the cell cycle, PANC-1 cells were stained with PI and measured by flow cytometry. When comparing cell cycle arrest results, SubG1 (90.38%) in the OJB-treated group was significantly higher than that in the control group (64.46%) (Figure 11). These results indicate that OJB effectively arrests the cell cycle in the G1/S phase. A major mechanism of cell cycle regulation depends on the activation of CDK-cyclin complexes [49,50]. Both CDK2 and CDK4 were decreased in a concentration-dependent manner in OJB-treated PANC-1 pancreatic cancer cells (Figure 12), indicating that OJB inhibits cell division and growth by effectively arresting the cell cycle in the G1/S phase. These results are consistent with those seen in flow cytometry.

5. Conclusion

This study confirmed that OJB, containing kaemepferol-3,7-diglucoside and kaempferol-3-rhamnosyl-7-glucoside with cynaroside as the main component, showed notable free radical scavenging

activity, anti-oxidant activity, and anti-inflammatory activity. In addition, this study showed that OJB, not only, significantly induces apoptosis of pancreatic cancer cells through both the intrinsic and extrinsic pathways, but also, induces cell cycle arrest in the G1/S phase to suppress cell division and proliferation, thereby effectively exhibiting anti-pancreatic cancer activity.

Conflict of Interest Statement

Authors declare no conflict of interest.

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Authors' Contributions

SHK was involved in designing the work, data collection, data analysis, and interpretation. DSL was involved in drafting the article.

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