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5,7,8-trihydroxyflavone Has Anticancer and Apoptotic Effects in Human Androgen-Independet Prostate Cancer PC-3 Cells

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Abstract: Natural products have been studied to provide alternative treatments against human diseases as they have various medicinal properties. One of these natural products is substances in the class of flavonoids. These bioactive molecules have antioxidant, anti-inflammatory and antitumor activities. The number of studies focusing on these molecules is increasing to discover new therapeutic agents against diseases such as cancer. We aimed to determine the anticancer and apoptotic effects of plant-derived natural 5,7,8-trihydroxyflavone (Nor-wogonin) on androgen-independent human prostate cancer (PC-3) cells *in vitro*. Nor-wogonin concentrations of 10, 20, 40, 80 µM were prepared and applied to human prostate cancer cells for 24 hours. The anticancer effect of flavone was determined by MTT 3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide) method, and its effect on pro-apoptotic and anti-apoptotic genes was determined by Real-Time PCR analysis. According to the obtained data, Nor-wogonin applied to PC-3 cells decreased *in vitro* cell viability due to increasing concentration ($p<0.05$) and the IC_{50} value was calculated as 57.29 µM. In addition, it was determined that Nor-wogonin directed PC-3 cells to apoptosis by acting on various anti-apoptotic and pro-apoptotic gene expressions.

Keywords: Prostate cancer; Nor-wogonin; Anticancer effect; Apoptosis

1 Introduction

Cancer is a serious health problem that accounts for 70% of all deaths worldwide (Cadona et al. 2022). Prostate cancer is the most common aggressive cancer among men (Barsouk et al. 2022). Aggressive and metastatic prostate cancer is among the dangerous, life-threatening tumors and is one of the leading causes of death in men (Apostolatos et al. 2018). Prostate tumors usually occur as a mixture of androgen-dependent and androgen-independent cells (Tang and Porter 1997; Russell et al. 1998). In advanced stage and metastatic prostate cancers, a fatal hormone-resistant disease develops with the growth of tumor cells and the formation of new cells independent of androgen. In this regard, it is important to introduce new treatment strategies and to encourage cells to apoptosis in the treatment of prostate cancers (Huang et al. 2004).

Carcinogenesis is a complex phenomenon involving genetic and epigenetic changes affecting tumor suppressor genes and oncogenes (Shu et al. 2010). In cancer treatment, natural products are becoming important candidates for drug discovery. Especially the phytochemicals in these products have been extensively researched as anticancer therapeutics. These bioactive molecules exert their anticarcinogenic activities by interfering with the formation, development and progression of cancer by regulating cell differentiation, proliferation, angiogenesis, metastasis and apoptosis (Chen et al. 2008). Many successful anticancer drugs currently available contain phytochemicals (Mondal et al. 2011) and some are being investigated in human clinical trials (Sultana 2011).

Flavones constitute one of the subgroups of flavonoids, which are among the most important bioactive compounds among secondary metabolites (Martens and Mithofer 2005). Flavonoids make up the largest group of polyphenols and are abundant in a variety of plant products such as fruits, and vegetables (Pallauf et al. 2017). Besides having biochemical and pharmacological activities, including antioxidant, anticarcinogenic, anti-inflammatory, antiproliferative and antiangiogenic effects, flavones do not have any toxic effects (Havsteen et al. 2002). It has been reported that flavonoids exert their antitumor effects through the induction of apoptosis, some phytochemicals such as tea, zerumbon and

turmeric have been investigated to trigger apoptosis by regulating the down-regulation of Bcl-2 and Bcl-XL in various tumor cells (Aggarwal et al. 2005; Takada et al. 2005; Nishikawa et al. 2006).

Nor-wogonin (5,7,8-trihydroxyflavone) is a polyhydroxy flavone isolated from *Scutellaria baicalensis* with various biological activities such as anticancer, antiviral and antioxidant (Hui et al. 2002; Miyasaki et al. 2013). Nor-wogonin induces apoptosis in HL-60 leukemia cells (Chow et al. 2008) and inhibits cell division by stopping the cell cycle in MDA-MB-231 cell line, leading cells to apoptosis and perform an antiproliferative effect (Abd El-Hafeez et al. 2019). In addition, Nor-wogonin has the potential to inhibit the growth of colorectal cancer cells *in vitro* by triggering apoptosis, autophagy and cell cycle arrest and it is thought to be developed as a possible anticancer agent (Wang et al. 2020).

It is important to define new therapeutics in cancer treatment and to reveal their mechanisms of action in cancerous cells (Eastman et al. 2006). Many studies conducted for this purpose focus on the discovery or development of new therapeutics (Genc et al. 2016, Sahin et al. 2018). In this study, we aimed to determine the anticancer and apoptotic effects of Nor-wogonin on the human prostate (PC-3) cancer cell line.

2 Materials and Method

2.1 Cell line, Cell Culture and Test Compound

Androgen-independent prostate cell PC-3 cells were used in the study. RPMI-1640 medium (Sigma-Aldrich, USA; added into the medium 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin) was used to feed PC-3 cells. Cells were fed in cell culture flask (TPP; Switzerland) and placed in a humidified incubator (5% CO2, 37°C; N-Biotek, Korean). The cell medium was refreshed on average every 3-4 days and cell passages were performed when the cells reached 90% confluence. The 10, 20, 40, 80 µM concentrations of Nor-wogonin (Cayman Chem, Cas number: 4443-09-8) in RPMI-1640 (for PC-3 cells) medium were prepared.

2.2 Determination of Cell Viability

The cytotoxic effect of Nor-wogonin on PC-3 cell line was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide) assay. Cells reaching 90% density were removed from the bottom of the flasks by Trypsin-EDTA solution and counted under an inverted microscope. Cells were seeded into 96-well microplates with $15x10^3$ cells per well. The seeded cells were incubated for 24 h in a CO_2 incubator at 37°C. After incubation, the media in the wells were refreshed, then different concentrations of Nor-wogonin were added to the microplate wells and incubated for 24 hours (Koran et al. 2017).

Then, the cell medium in the microplate was removed and the MTT solution (0.5 mg/mL) was added to each well and incubated for 3 h. After the incubation, MTT solution in the wells was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to each well and the incubation was stopped. Optical densities of cells in microplates were determined by spectrophotometer (Thermo, USA) at a wavelength of 570 nm (Mosmann 1983).

The average of the absorbance values obtained from the control wells was calculated and this value was accepted as 100% viable cells. The percentages of viability values in the experimental wells were determined by comparing the absorbance values obtained from Nor-wogonin applied wells to the control absorbance value.

2.3 Determination of the IC_{50} Value

According to the MTT analysis data, it was determined at what dose the high level effect of Nor-wogonin occurred. The effects of the compounds at different applied concentrations were evaluated using the GraphPad Prism 9 program to calculate the inhibitory concentration 50 (IC_{50}) value of the test compound on PC-3 cells. In the studies to be carried out after this step, the determined effective doses were applied to the cells.

2.4 RNA Isolation and Complementary DNA (cDNA) Synthesis

For the total RNA isolation, 1.5 mL of cold PBS was added to each well of 6-well microplates containing 1x106 cells treated with Nor-wogonin IC_{50} values for both PC-3 cells and then cells were scraped from the bottom of the flask. Total RNA isolation from human PC-3 cell line performed in accordance with the manufacturer's protocol (Thermo Fisher Scientific). cDNA synthesis was performed with the following the manufacturer's protocol (Bio-Rad).

2.5 Primer design and gene expression analysis

Polymerase chain reactions (PCR) were performed on a Bio-Rad CFX96 Real-Time PCR cycler and with a master mix (2xqPCRBIO SyGreen Mix Lo-ROX Kit, PCR Biosystems) containing SYBR Green, which was used to obtain gene expression profiles of cDNA samples. The associated genes of primers sequences were designed by Primer3 software program (v. 0.4.0), and they were obtained commercially (Metabion). The specific binding forward and reverse primer sequences of the associated gene and gene symbols are given in Table 1. β-Actin was taken as a housekeeping gene in qPCR studies.

qPCR reaction conditions; after incubation at 95°C for a period of 2 minutes; at 95°C for a period of 5 sec, 40 cycles; at 66°C for a period of 45 sec, 45 cycles; it was set at 74°C for a period of 2 minutes, 45 cycles, and finally at 72°C for a period of 5 minutes, 1 cycle. mRNA expression rates were determined by the comparative threshold cycle (2-ΔΔCt) method (Livak and Schmittgen; 2001). Ct values were normalized with β-actin mRNA expression ratios (Livak and Schmittgen 2001).

Table 1. Forward and reverse primer sequences designed for use in quantitative polymerase chain reaction.

Gene names	Primer Sequences (5'-3')
β-actin	Forward CACCCCAGCCATGTACGTTGC
	Reverse CCAGCCCATGATGGTTCTGAT
bcl-2	Forward GAGGGGCTACGAGTGGGATGC
	Reverse GGAGGAGAAGATGCCCCGGTGC
bax	Forward CCCGAGAGGTCTTTTTCCGAG
	Reverse CCAGCCCATGATGGTTCTGAT
p53	Forward CCTCAGCATCTTATCCGAGTGG
	Reverse GGATGGTGGTACAGTCAGAGC
caspase-3	Forward GCGAATCAATGGACTCTGGAA
	Reverse GTCAACAGGTCCATTTGTTCC

2.6 Statistical analysis

Statistical analyzes were performed with the GraphPad Prism 9 package program. One-way ANOVA was used to detect changes between different groups and Tukey's test was used for multiple comparisons. Quantitative data were analyzed as mean with standard deviation (mean±SD). The degree of significance of the analyzes was given with the symbol (*). p>0.05 (not significant, ns); *p<0.05 (significant); **p<0.01 (very significant); ***p<0.001 (highly significant).

3 Results and Discussion

The cytotoxic effect of Nor-wogonin on the human prostate cancer cell line is shown in Fig. 1. Accordingly, the applied 10 and 20 µM concentrations of flavone did not reveal a significant change in cell viability compared to the control group, while the 40 and 80 µM concentrations significantly decreased the cell viability (*p<0.05). In addition to cell viability assay we detected the IC_{50} values of the Nor-wogonin. The IC_{50} values of Nor-wogonin on PC-3 cells were calculated as 57.29 µM.

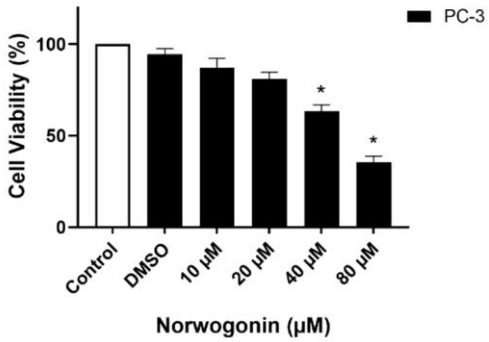

Fig. 1 The % change in viability of PC-3 human prostate cancer cells treated with different concentrations of Nor-wogonin for 24 hours. The data obtained are shown as mean±SD. *p<0.05 vs control group.

The mRNA expression levels of bcl-2, bax, p53 and caspase-3 genes of Nor-wogonin applied to PC-3 and HeLa cells were determined. The mRNA expression levels of the genes were calculated by the $2^{-\Delta\Delta CT}$ method (Livak KJ and Schmittgen 2001). The results were normalized using the mRNA expression level of the β-actin reference gene. mRNA expression rates of bcl-2, bax, p53 and caspase-3 genes were determined. The mRNA expression levels of all genes were evaluated as 1.0 for the control group and the results were given as fold values compared to the control group. The rates of increase and decrease of mRNA expressions of bcl-2, bax, p53 and caspase-3 genes compared to control groups PC-3 cells are given respectively in Fig. 2.

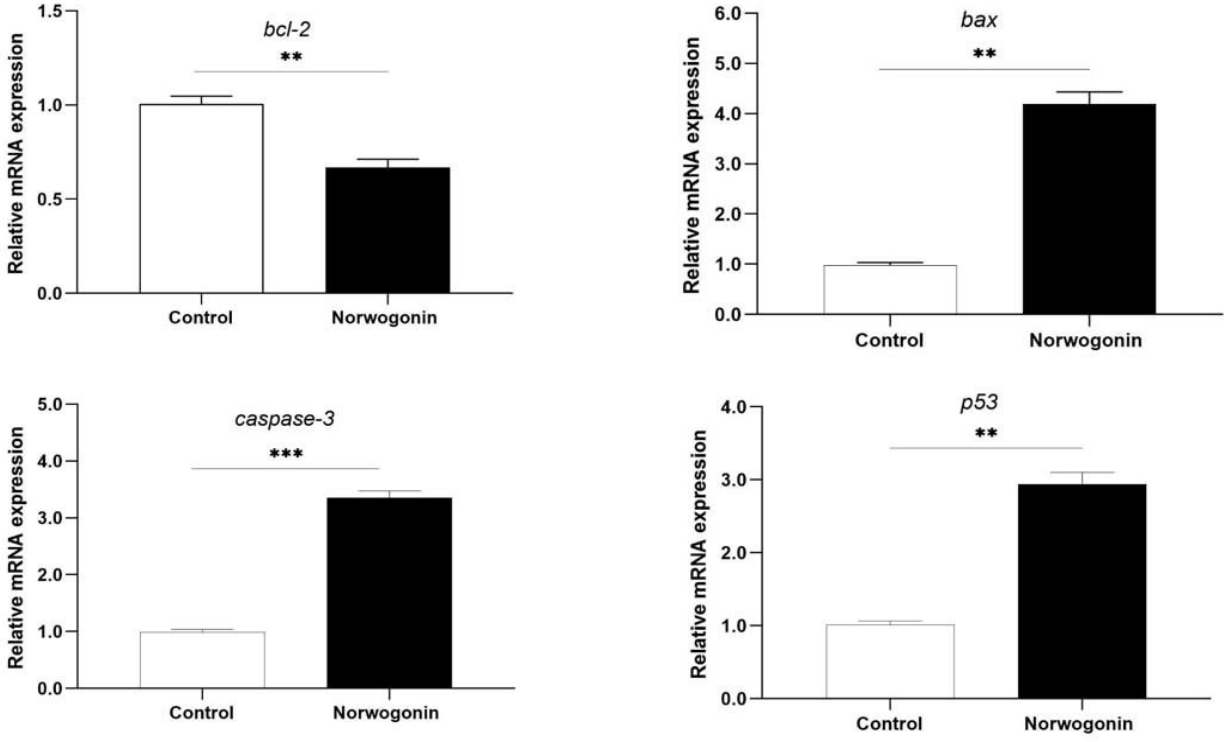

Fig.2 Quantitative changes in the gene expression levels of the control and Nor-wogonin groups in PC-3 cells. Changes in the gene expression levels of bax, bcl-2, p53 and caspase-3 (d) were examined by qPCR. β-Actin was used as a housekeeping gene. mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction.

Prostate cancers are a group of tumors associated with androgen hormone (Panvichian and Pienta 1996, Klocker et al. 1999). Androgen deprivation and estrogen hormone replacement therapies have attracted attention as treatment strategies to reduce the growth of these types of tumors. However, androgen ablation may lead to the development of androgen-independent tumors that are less responsive to conventional chemotherapeutic treatments (Panvichian and Pienta 1996; Klocker et al. 1999).

Studies indicate the availability of herbal-based flavonoid therapies as an alternative method to arrest androgen-independent tumor growth. How flavonoids alter the growth of these types of cells has not been determined, but suggests that they alter the estrogen receptor and expression of hormone-sensitive genes (Denis et al. 1999).

Natural products of plant origin, such as flavonoids, are the leading sources of many medicines and dietary supplements (Hui et al. 2002, Li and Vederas, 2011). The biological effects of Wogonin and Nor-wogonin, which are polyhydroxyflavones and are relatively similar in structure, have been reported (S Patel et al. 2013). The structural difference between Wogonin and Nor-wogonin is due to the presence of a methoxyl (OCH3) group on the C8 of Wog and a hydroxyl (OH) group on the C8 of N-Wog (Huang et al. 2017). Nor-wogonin has been reported to be a more effective apoptotic inducer than Wogonin in human leukemia HL-60 cells (Chow et al. 2008). In another study, it was reported that Nor-wogonin had no toxic effect on rat PC-12 normal adrenal gland cells (Jing et al. 2021). There are not enough studies in the literature with the cytotoxic activity of Nor-wogonin compared to Wogonin. Therefore, in this study, we aim to contribute to the literature by investigating the cytotoxic effect of Nor-wogonin in human androgen-independet prostate cancer cells.

In the studies, the anticancer effect of Nor-wogonin and the IC_{50} values in androgen-independented prostate cancer cells were calculated. In one of these studies, Nor-wogonin was reported to decrease the viability of human triplet breast cancer cells, and the IC_{50} values of Nor-wogonin in MDA-MB-231, HCC70, BT-549, and HCC1806 were 32,24, 39.05, 56.2, and 37.3 μM, respectively (Abd El-Hafeez et al. 2019). In another study, it was emphasized that Nor-wogonin had a cytotoxic effect on HL-60 human leukemia and SW48 human colon cancer cells, and the IC_{50} values of HL-60 and SW48 cells were calculated as 21.7 μM and 15.5 μM, respectively (Chow et al. 2008). In our previous study, we also showed the anticancer effect of Nor-wogonin in human cervical cancer HeLa cell line and determined the IC_{50} value as 32.09 μM (Karakuş and Ünal Karakuş, 2022). According to our findings in this study, Nor-wogonin decreased cell viability in PC-3 cells. While a statistically significant decrease in cell viability was observed at 40 and 80 μM concentrations of Nor-wogonin treated with PC-3 cells, no statistically significant decrease was detected at 10 and 20 μM concentrations. In addition, the IC_{50} value of Nor-wogonin for PC-3 cells was calculated as 57.29 μM.

Natural compounds such as flavonoids are considered important agents for the prevention and treatment of cancer due to their potential therapeutic effects and limited toxicity to healthy cells (Toshiya et al. 2012). In carcinogenesis, flavonoids interfere with intracellular signal transduction pathways, thereby suppressing proliferation, metastasis, angiogenesis and increasing apoptosis (Ravishankar et al. 2013, Srivastava et al. 2016). Controlled by genes in the cell, apoptosis is part of the cell's active death process (Kerr et al. 1972). In our study, we also investigated the expression levels of antiapoptotic bcl-2, proapoptotic bax, caspase-3 and p53 genes, which are involved in apoptotic processes of Nor-wogonin in PC-3 cells. While bcl-2 gene expression decreased in PC-3 cells, the expression of bax, caspase-3 and p53 genes increased.

Activation of the caspase cascade occurs in cells undergoing apoptosis (Estaquier et al. 2012). In this study, Nor-wogonin increased caspase-3 gene expression in both cell lines. Bax and bcl-2 gene expression rate is important in initiating apoptosis (Liu et al. 2005). According to our findings, Nor-wogonin increased the expression rate of bcl-2/bax genes in PC-3 cells.

Tumor suppressor protein p53 is responsible for regulating bax and bcl-2 gene expression, activating cell cycle checkpoints, DNA repair mechanisms and apoptosis response (Farnebo et al. 2010). DNA-damaging agents induce increased p53, which plays a leading role in directly activating the pro-apoptotic bax gene to activate the apoptotic program (Miyashita et al. 1994). In this study, Nor-wogonin increased p53 expression in PC-3 cells.

5 Conclusion

Nor-wogonin showed anticancer effect in PC-3 human prostate cancer cells and induced apoptosis by showing increased bax, caspase-3 and p53 gene expression and decreased bcl-2 gene expression. However, further studies are needed to better understand how Nor-wogonin affects the molecular mechanisms in cell death and to determine its biocompatibility levels.

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Conflict of interest disclosure:

The authors of this study declare that they have no conflict of interest.

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