Anti-cancer effect of ethylacetate fraction from *Orostachys japonicus* on HT-29 human colon cancer cells by induction of apoptosis through caspase-dependent signaling pathway

Deok-Seon Ryu¹, Hyun-Ji Lee², Ji-Hye Kwon², Dong-Seok Lee²

¹Department of Biomedical Laboratory Science, Soonchunhyang University, Asan 31538, Republic of Korea
²Department of Smart Foods and Drugs, Graduate School of Inje University, Gimhae 50834, Republic of Korea

ARTICLE INFO

Article history:
Received 30 November 2017
Received in revised form 20 January 2018
Accepted 10 February 2018
Available online 1 May 2018

Keywords:
Orostachys japonicus
HT-29 human colon cancer cells
Anti-cancer activity
Apoptosis
Caspase cascade

ABSTRACT

Objective: To investigate the anti-colon cancer effects of ethylacetate fraction from *Orostachys japonicus* (*O*. *japonicus*) on HT-29 cancer cells. Methods: The viability of HT-29 cells was assayed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) method. Apoptosis induction and cell cycle inhibition were confirmed by fluorescein isothiocyanate and propidium iodide staining using flow cytometry. Morphological changes in the nucleus were observed, using a fluorescence microscope with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining. The expression levels of the upstream and downstream proteins involved in the anti-cancer mechanism were confirmed by Western blotting. Results: After treating HT-29 cells with different concentrations of ethylacetate fraction from *O*. *japonicus*, the viability of cells decreased in a concentration-dependent manner, while apoptosis induction and apoptotic body formation increased. Cell cycle analysis showed that the arrest occurred at the sub-G₁ and S phase. Among the upstream and downstream proteins involved in the anti-cancer mechanism were confirmed by Western blotting. Results: After treating HT-29 cells with different concentrations of ethylacetate fraction from *O*. *japonicus*, the viability of cells decreased in a concentration-dependent manner, while apoptosis induction and apoptotic body formation increased. Cell cycle analysis showed that the arrest occurred at the sub-G₁ and S phase. Among the upstream and downstream proteins involved in the anti-cancer activity, the level of B cell lymphoma-2 decreased, and the bcl-2-associated x protein increased. The level of pro-caspase-3, pro-caspase-8, and pro-caspase-9 decreased, while the level of cleaved-caspase-3, cleaved-caspase-8, and cleaved-caspase-9 increased. Moreover, the phosphorylation, that is, activation of extracellular signal regulated kinase 1/2, Jun-N-terminal kinase, and p38 increased. Conclusions: Combining the above results, it is thought that the survival of HT-29 cells is suppressed by ethylacetate fraction from *O*. *japonicus* through mitochondrial regulation-induced caspase cascade activation, induction of apoptosis and cell cycle arrest.

1. Introduction

Due to rapid industrial and economic development, irregular lifestyles, lack of exercise, and pollution, the rate of chronic diseases includes cancer, cardiovascular diseases, and mental illnesses is increasing each year. Among these chronic diseases, cancer is the primary cause of death globally. According to the updated data from cancer statistics in Korea in 2015, colon cancer is the third (12.13%) leading cause of death in Korea[1]. Although surgery, chemotherapy, and/or radiation therapy are used as conventional cancer treatments, these methods may lead to adverse side effects such as DNA damage, neural toxicity, and hair loss, which possibly hinder the successful treatment of the disease. Many recent studies...
have been conducted on natural substances that are safer and more effective than conventional methods. Apoptosis is an essential step in cell proliferation and the maintenance of homeostasis in multicellular organisms, blebbing, cell shrinkage, and DNA fragmentation[2-4]. Two well-known mechanisms of apoptosis are the mitochondria-mediated intrinsic apoptotic pathway and the death receptor mediated extrinsic apoptotic pathway. Inducing apoptosis in cancer cells has been thought to be crucial to the development of organisms and maintenance of tissues, as it leads to selective cell death. The initiation of apoptotic signaling pathway suppresses anti-apoptotic proteins for example B cell lymphoma-2 (bcl-2) is an anti-apoptotic protein. Decreasing the expression of bcl-2 has been shown to dramatically reduce the number of leukemic cells, making it a potentially very useful way for treating cancer[5-7]. In the intrinsic pathway, cytochrome c released from mitochondria binds pro-caspase-9 to activate it, and then, the activated cleaved caspase-9 then activates the effector pro-caspase-3 into cleaved caspase-3 which degrades cells[8-12]. Mitogen activated protein kinase (MAPK) is also known to be participated in induction of apoptosis according to several studies[13,14].

Orostachys japonicus (O. japonicus), a biennial herb plant which belongs to the family Crassulaceae and named as Wa-song in Korea, is used conventionally as a folk medicine for its activities of anti-inflammation, anti-febrility, hemostasis, antidote, and especially anti-cancer. Friedelin, epifriedelanol, glutinone, glutinol, β-amyrin, triterpenoid derivatives, campsterol, sterol glucosides, fatty acid esters, kaempferol, quercetin, flavonoid glycosides, 1-hexatriacontanol, and oxalic acid have been reported to be contained in this herb[15-23]. Furthermore, it has been shown to be effective in treating stomach cancer, liver cancer, and colon cancer and to have anti-microbial and anti-diabetic substances. In our studies, dried milled O. japonicus was collected with ethanol and categorized with organic solvents[20]. After identifying the ethylacetate fraction through gas chromatography–mass spectrometry, 12 peaks were taken. Among them, three peaks were identified as kaempferol (6.81%), quercetin (5.08%), and gallic acid (4.24%)[20,21]. These soluble fractions were examined for activities of anti-cancer, anti-inflammatory, and anti-oxidizing activities. Among these extracts, ethylacetate fraction exhibited the best activity for anti-cancer. Although previous reports have disclosed the effectivity of ethylacetate fraction in treating stomach cancer and liver cancer[19-24], its roles in induction of necessary for apoptosis, the cell cycle block, and intracellular signaling pathways in HT-29 colon cancer cells have remained uninvestigated. In this study, we provide some solid evidences to demonstrate the mechanism of the anti-colon cancer activity of the ethylacetate fraction derived from O. japonicus.

2. Materials and methods

2.1. Cell Line and reagents

The ethylacetate fraction was extracted from O. japonicus (OJEF for short) in our laboratory using methods described previously[20-25]. HT-29 cells were gained from the Korean cell line bank (KCLB; Seoul, Korea). DMEM, 10% FBS, antibiotic solution, monoclonal antibodies such as bcl-2, bax, caspase-3, -8, -9, cleaved caspase-3, -8, -9, phospho-MAPK, MAPK, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and secondary antibody against goat anti-rabbit IgG-HRP were prepared from the same suppliers as described previously[20-25].

2.2. Cell culture

The HT-29 cells were sub-cultured once 5 days. The medium was replaced every 3 days. When cultures were 85%--95% confluent, they were used for experiments. The OJEF solution mixed with 0.1% dimethyl sulfoxide (Sigma-Aldrich, MO, USA) was used to treat the cells.

2.3. Cell viability assay

The CellTiter 96 Non-radioactive Cell Proliferation Assay Kit (Promega, WI, USA) was used to assess cell viability. The cells were seeded in 96-round well plate and treated with OJEF. After complete removing of the medium and washing with PBS, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was added. The plate was placed at 37 °C for 1 h, and then, the parameter was analyzed at 490 nm using an ELISA reader (PowerWaveXS, BioTek, VT, USA).

2.4. Flow cytometric analysis of apoptosis

An Annexin V-FITC Apoptosis Detection kit (BD Biosciences, NJ, USA) was used to assess the apoptotic rate of HT-29 cells. The cells were seeded in 24-round well plate and treated with OJEF. After 12 h, the medium was completely removed and washed. Binding buffer was added, subsequently applying with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) reagent. The reaction was allowed to take place in the dark for 15 min, and then the samples were analyzed using flow cytometry (Becton Dickinson, NJ, USA).

2.5. Flow cytometric analysis of cell cycle

A Cell Cycle Phase Determination kit (Cayman Chemical, MI, USA) was used to assess the cell cycle division. The HT-29 cells were dispensed into 24-round well plate and then treated with OJEF.
After 12 h, the medium was completely removed and washed. Then, the assay buffer was applied. The cells were fixed using 100% ethanol for at least 2 h. The fixing solution was then completely removed, and the cells were suspended in sufficient amount of RNase-containing PI staining solution. The reaction was allowed to occur in the dark place. The samples were analyzed by flow cytometry.

2.6. Nuclear staining assay

The HT-29 cells incubated for 12 h were suspended in Triton-X-containing PBS solution. They were centrifuged; the cell pellets were suspended in 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA, USA) staining solution. Subsequently, 20 µL of the suspension was dropped on glass slide and covered with a cover glass, such that the bubbles were avoided. This was incubated at cold temperature overnight. Morphological changes in the nucleus were checked by fluorescence microscope (LSM510 Meta, Carl Zeiss, Jena, Germany).

2.7. Western blotting

The HT-29 cells were dispensed into 6-round well plate and then treated with OJEF. Cold PBS was completely removed, and the proteins were collected using lysis buffer. Protein concentrations were calculated using a BCA protein Assay kit (Thermo scientific, IL, USA). Testing materials were prepared by mixing the quantified proteins and loading buffer in a 1:1 ratio and heating them at 95 °C for 5 min. The samples prepared at different concentrations were electrophoresed on a 10%–15% SDS–PAGE, and then transferred to a PVDF membrane using a semi-dry method (Bio-Rad, CA, USA). The membrane was blocked using 5% non-fat milk at 4 °C for at least 4 h, and then incubated with a primary antibody at 4 °C overnight. The membrane was then washed with PBST thrice and treated with a secondary antibody for approximately 2 h. The fluorescent protein bands were prepared with a luminol reagent, and developed in a darkroom using a film.

2.8. Statistical analysis

All experiments were carried out at least 3 times. The statistical differences were calculated in the Student’s t test using the SigmaStat software (SigmaStat for Windows Version 3.0.). \( P<0.05 \) was regarded statistically meaningful.

3. Results

3.1. Inhibition of cell growth

The proliferation of HT-29 cells treated with different concentrations of OJEF was assayed with MTS method. The HT-29 cells were treated with OJEF at concentrations of 25, 50, 75, and 100 µg/mL and incubated for 12 and 24 h. After 12-hour incubation, the respective cell viabilities were \((66.84±6.30)\%\), \((58.79±15.70)\%\), \((34.86±5.70)\%\), and \((30.02±6.20)\%\). After 24-hour postincubation, the respective cell viabilities were \((62.94±21.13)\%\), \((46.77±32.20)\%\), \((31.08±29.71)\%\), and \((17.19±19.21)\%). The proliferation of OJEF-treated HT-29 cells decreased in a both concentration- and time-dependent manner. These results show that OJEF suppress the proliferation of HT-29 cells.

3.2. Induction of apoptosis

The HT-29 cells treated with OJEF for 12 h were stained with annexin V-PI to investigate the rates of cell apoptosis, and the apoptosis rates for the OJEF untreated control were \((3.22±1.93)\%\) for early apoptosis and \((5.22±5.03)\%\) for late apoptosis. However, the rates for the group treated with 100 µg/mL OJEF were \((14.15±5.01)\%\) for early apoptosis and \((16.98±2.82)\%\) for late apoptosis. Besides, the respective rates of early apoptosis treated with different concentrations of OJEF [0 (0.1% DMSO, control), 25, 50, 75, and 100 µg/mL] for 12 h were \((3.22±1.93)\%\), \((4.50±2.36)\%\), \((6.48±0.39)\%\), \((8.56±2.12)\%\) and \((14.15±5.01)\%). The respective rates of late apoptosis were \((5.22±5.03)\%\), \((7.07±8.51)\%\), \((9.08±7.62)\%\), \((12.90±1.93)\%\) and \((16.98±2.82)\%\). These results showed elevated levels of early and late apoptosis in the OJEF-treated group in a concentration-dependent manner compared to the control group. Based on the result of the DAPI nuclear staining, which was performed to observe the apoptotic rate, notable morphological changes in the nucleus, such as apoptotic body formation, were observed by the naked eye; these changes in the OJEF-treated group were found to occur in a concentration-dependent manner (Figure 1).

3.3. Induction of cell cycle arrest

To determine if there is a correlation between the anti-proliferation
effect of OJEF on HT-29 cell growth and the apoptotic effect, we examined the cell cycle using flow cytometry. In the sub-G₁ phase, which is an evidence of apoptosis (Table 1), treatment with 100 µg/mL of OJEF resulted in a (35.7±5.57)% arrest, in contrast to the control, which showed a (1.03±0.81)% arrest, thereby confirming an arrest in cell cycle progression. Similarly, with regards to the S phase, treatment with 100 µg/mL of OJEF resulted in a (11.68±2.36)% arrest, in contrast to the control, which showed a (5.2±1.15)% arrest, thereby confirming the increased impediment of cell cycle advance. The S phase cell proportion of the OJEF-treated cells increased in a concentration-dependent way. The HT-29 cells treated with OJEF caused manifest block of cell cycle in the sub-G₁ and S phase.

### Table 1

| Group | Sub-G₁ | G₁       | S       | G₂/M  |
|-------|--------|----------|---------|-------|
| Control | 1.03±0.81 | 76.97±2.96 | 5.20±1.15 | 15.44±2.83 |
| 25 µg/mL OJEF | 4.81±2.36 | 68.78±2.80 | 8.30±1.53 | 18.47±0.23 |
| 50 µg/mL OJEF | 10.39±5.35 | 65.90±6.93 | 10.19±4.65 | 13.84±2.12 |
| 75 µg/mL OJEF | 31.13±9.98 | 48.15±8.32 | 9.78±1.27 | 11.45±2.74 |
| 100 µg/mL OJEF | 35.73±5.57 | 44.99±6.66 | 11.68±2.36 | 8.15±0.46 |

The results are shown as the mean±SD. Cells treated only with 0.1% DMSO was used as control. *P<0.05 was regarded statistically meaningful (vs. control).

### 3.4. Apoptosis-related downstream signaling pathways

Among the pro-apoptosis and anti-apoptosis factors, the correlation between the representative apoptosis-inhibiting factor bcl-2 and apoptosis-promoting factor bax was confirmed by their expression levels, which were measured by Western blotting. Table 2 shows bcl-2 decreased while bax increased in an OJEF concentration-dependent way. These results indicate that OJEF promoted apoptosis in HT-29 cells through the mitochondria-mediated pathway. Caspases proteins are integral proteases that induce cell apoptosis in relation to bcl-2 and bax proteins in downstream signaling pathways. Caspas are divided into three types according to structural differences: caspase-3, caspase-8, and caspase-9, and their roles also differ. To analyze the expression level of caspases during the cascade of activation Western blotting was executed. Cells were treated with concentrations of OJEF [0 (0.1% DMSO, control), 25, 50, 75, and 100 µg/mL] for 12 h. The expression of inactive forms such as pro-caspase-3, pro-caspase-8, and pro-caspase-9 decreased in the OJEF treatment group except 50 µg/mL OJEF group (Table 2). In contrast, the expression of the active forms of cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 increased in the OJEF treatment group except cleaved caspase-8 in 100 µg/mL OJEF group.

### Table 2

| Group | Bcl-2 | Bax | Pro-caspase-3 | Pro-caspase-8 | Pro-caspase-9 | Cleaved caspase-3 | Cleaved caspase-8 | Cleaved caspase-9 |
|-------|-------|-----|--------------|--------------|--------------|------------------|------------------|------------------|
| Control | 0.97±0.49 | 0.23±0.30 | 1.59±0.48 | 0.94±0.24 | 1.47±0.02 | 0.54±0.44 | 1.07±0.26 | 0.30±0.20 |
| 25 µg/mL OJEF | 0.67±0.33 | 0.46±0.19 | 1.39±0.83 | 1.02±0.27 | 1.31±0.07 | 0.69±0.32 | 1.28±0.25 | 1.02±0.82 |
| 50 µg/mL OJEF | 0.47±0.23 | 1.07±0.62 | 2.22±0.45 | 1.08±0.31 | 1.40±0.09 | 1.24±0.86 | 1.45±0.27 | 1.49±1.08 |
| 75 µg/mL OJEF | 0.50±0.25 | 1.66±0.89 | 1.59±0.99 | 1.00±0.28 | 0.99±0.14 | 1.45±0.21 | 1.25±0.39 | 0.93±0.40 |
| 100 µg/mL OJEF | 0.25±0.19 | 1.87±0.80 | 0.98±0.65 | 0.15±0.09 | 0.77±0.09 | 1.60±0.42 | 0.68±0.37 | 0.90±0.26 |

The results are shown as the mean±SD. Ratio of protein levels was observed by Image J program. The density of bands between the target protein and GAPDH was used as a housekeeping gene. *P<0.05 was regarded statistically meaningful (vs. control).

### 3.5. Apoptosis-related upstream signaling pathway

To check if there is a correlation between the downstream mitochondria-mediated apoptosis pathways and upstream signaling pathways, we confirmed the MAPKs that are involved in directing the upstream signaling transduction system related to apoptosis using Western blotting. As shown in Table 3, the phospho-extracellular signal regulated kinase 1/2 (ERK1/2), Jun-N-terminal kinase (JNK), and p38 increased compared with the total form. In contrast, the total form of ERK1/2, JNK, and p38 did not change.

### Table 3

| Group | Phospho-ERK1/2 | ERK1/2 | Phospho-JNK | JNK | Phospho-p38 | p38 |
|-------|----------------|--------|-------------|-----|------------|-----|
| Control | 0.54±0.48 | 0.45±0.46 | 0.76±0.14 | 1.40±0.30 | 0.31±0.11 | 1.41±0.25 |
| 25 µg/mL OJEF | 0.57±0.36 | 2.46±0.69 | 1.71±0.22 | 1.30±0.27 | 0.30±0.30 | 1.36±0.39 |
| 50 µg/mL OJEF | 1.16±0.73 | 2.56±0.57 | 1.53±0.53 | 1.35±0.36 | 0.31±0.23 | 1.40±0.32 |
| 75 µg/mL OJEF | 1.69±1.05 | 2.53±0.69 | 1.46±0.81 | 1.47±0.28 | 0.48±0.41 | 1.38±0.31 |
| 100 µg/mL OJEF | 1.55±0.89 | 2.41±0.67 | 2.44±0.31 | 1.40±0.28 | 0.64±0.43 | 1.39±0.36 |

The results are shown as the mean±SD. Ratio of protein levels was observed by Image J program. The density of bands between the target protein and GAPDH was calculated. Cells treated only with 0.1% DMSO was used as control. GAPDH was used as a housekeeping gene. *P<0.05 was regarded statistically meaningful (vs. control). ERK1/2: extracellular signal regulated kinase 1/2; JNK: Jun-N-terminal kinase.

### 4. Discussion

In this study, experiments were performed to investigate how the biological active substance OJEF affects the up-regulation...
and down-regulation of cell signaling pathways that are directly related to cancer development, suppresses cancer cell proliferation, and regulates the cell cycle[3]. After the initiation of apoptosis, phosphatidylinositol is exposed on the cellular membrane, which inhibits anti-apoptotic factors such as bcl-2 and activates pro-apoptotic factors such as bax as caspases. Furthermore, in the nucleus, the activation of endonuclease leads to DNA fragmentation, chromatin condensation, destruction of the nuclear membrane, and blebbing of the nucleus. In cancer, there is an imbalance between cell proliferation and death that results in the continued multiplication of cells. Therefore, control of the cell cycle is a pivotal factor in the study of anti-cancer activity[15,20].

In addition, other studies published to date on whole plants, soluble extracts, identifying potential active matters and their anti-bacterial, anti-viral factor in the study of anti-cancer activity. In the study, it is more likely that anti-cancer property of OJEF is controlled by the mitochondria-mediated pathway regulated by bcl-2 family proteins, ending with the cleavage of pro-caspase-3 to execute programmed cell death by chromatin condensation and formation of apoptotic bodies. In addition, OJEF sends stress signals to HT-29 colon cancer cells, resulting in the activation of MAPK family proteins and the induction of a cascade representing an anti-cancer mechanism through the phosphorylation of ERK, JNK, and p38.

Conflict of interest statement
All authors declare that there are no conflicts of interest.

Acknowledgments
This work was supported by the 2016 Inje University research grant.

References
[1] National Cancer Information Center Korea. Cancer statistics reports for the Korea. [Online]. Available from: http://www.cancer.go.kr/mbs/cancer/fплпл/ [Accessed on December 9, 2014].
[2] Han SI, Kim YS, Kim TH. Role of apoptotic and necrotic cell death under physiologic conditions. BMB Rep 2008; 41(1): 1-10.
[3] Seo JA, Kim B, Dhanasekaran, DN, Tsang BK, Song YS. Curcumin induces apoptosis by inhibiting sarcoplasmic reticulum Ca^{2+} ATPase activity in ovarian cancer cells. Cancer Let 2016; 371(1): 30-37.
[4] Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 2010; 48(6): 749-762.
[5] Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. Nat Rev Cancer 2008; 8(3): 159-175.
[6] Matt S, Hofmann TG. The DNA damage-induced cell death response: A roadmap to kill cancer cells. *Cell Mol Life Sci* 2016; 73(15): 2829-2850.

[7] Fríón-Herrera Y, Díaz-García A, Ruiz-Fuentes J, Rodríguez-Sánchez H, Sforcin JM. Brazilian green propolis induced apoptosis in human lung cancer A549 cells through mitochondrial-mediated pathway. *J Pharm Pharmacol* 2015; 67(10): 1448-1456.

[8] Jin UH, Song KH, Motomura M, Suzuki I, Gu YH, Kang YJ, et al. Caffeic acid phenethyl ester induces mitochondria-mediated apoptosis in human myeloid leukemia U937 cells. *Mol Cell Biochem* 2008; 310(1-2): 43-48.

[9] Bhola PD, Letai A. Mitochondria-judges and executioners of cell death sentences. *Mol Cell* 2016; 61(5): 695-704.

[10] Jiang AJ, Jiang G, Li LT, Zheng JN. Curcumin induces apoptosis through mitochondrial pathway and caspases activation in human melanoma cells. *Mol Biol Rep* 2015; 42(1): 267-275.

[11] El Maadidi S, Faletti L, Berg B, Wenzl C, Wieland K, Chen ZJ, et al. A novel mitochondrial MAVS/Caspase-8 platform links RNA virus-induced innate antiviral signaling to Bax/Bak-independent apoptosis. *J Immunol* 2014; 192(3): 1171-1183.

[12] Su Z, Yang Z, Xu Y, Chen Y, Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol Cancer* 2015; 14(1): 48.

[13] Sebolt-Leopold JS. Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene* 2000; 19: 6594-6599.

[14] Wang J, Yuan L, Xiao H, Wang C, Xiao C, Wang Y, et al. A novel mechanism for momordin Ic-induced HepG2 apoptosis: Involvement of PI3K- and MAPK-dependent PPARγ activation. *Food Funct* 2014; 5(5): 859-868.

[15] Lunghi P, Giuliani N, Mazzera L, Lombardi G, Ricca M, Corradi A, et al. Targeting MEK/MAPK signal transduction module potentiates ATO-induced apoptosis in multiple myeloma cells through multiple signaling pathways. *Blood* 2008; 112(6): 2450-2462.

[16] Zhang YX, Kong CZ, Wang HQ, Wang LH, Xu CL, Sun YH, et al. Phosphorylation of Bcl-2 and activation of caspase-3 via the c-Jun N-terminal kinase pathway in ursolic acid-induced DU145 cells apoptosis. *Biochimie* 2009; 91(9): 1173-1179.

[17] Sun Y, Liu, W Z, Liu T, Feng X, Yang N, Zhou HF. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J Recept Signal Transduct Res* 2015; 35(6): 600-604.