Natural compounds, fraxin and chemicals structurally related to fraxin protect cells from oxidative stress

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Abbreviations: CAT, catalase; DCFH-DA, 2',7'-dichlorodihydrofluorescein-diacetate; DDRT-PCR, differential display reverse transcription-PCR; GPX, glutathione peroxidase; HUVECs, human umbilical vein endothelial cells; LPO, lipid peroxidation; MDA, malondialdehyde; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase

Abstract

Coumarins comprise a group of natural phenolic compounds found in a variety of plant sources. In view of the established low toxicity, relative cheapness, presence in the diet and occurrence in various herbal remedies of coumarins, it appears prudent to evaluate their properties and applications further. The purpose of this study is to investigate cellular protective activity of coumarin compound, fraxin extracted from Weigela florida var. glabbra, under oxidative stress, to identify genes expressed differentially by fraxin and to compare antioxidative effect of fraxin with its structurally related chemicals. Of the coumarins, protective effects of fraxin against cytotoxicity induced by H₂O₂ were examined in human umbilical vein endothelial cells (HUVECs). Fraxin showed free radical scavenging effect at high concentration (0.5 mM) and cell protective effect against H₂O₂-mediated oxidative stress. Fraxin recovered viability of HUVECs damaged by H₂O₂-treatment and reduced the lipid peroxidation and the internal reactive oxygen species level elevated by H₂O₂ treatment. Differential display reverse transcription-PCR revealed that fraxin upregulated antiapoptotic genes (clusterin and apoptosis inhibitor 5) and tumor suppressor gene (ST13). Based on structural similarity comparing with fraxin, seven chemicals, fraxidin methyl ether (29.4% enhancement of viability), prenylketin (26.4%), methoxsalen (20.8 %), diffratic acid (19.9%), rutoside (19.1%), xanthyletin (18.4%), and kuhlmannin (18.2 %), enhanced more potent cell viability in the order in comparison with fraxin, which showed only 9.3% enhancement of cell viability. These results suggest that fraxin and fraxin-related chemicals protect HUVECs from oxidative stress.

Keywords: antioxidants; apoptosis; coumarins; fraxin; oxidative stress; plants, medicinal

Introduction

Coumarins comprise a very large class of phenolic substances found in plants and are made of fused benzene and α-pyrene rings. To date, at least 1,300 coumarins have been identified, principally as secondary metabolites in green plants but also in fungi and bacteria (Murray, 1989; Hoult and Paya, 1996). The prototypical compound is coumarin itself (also known as 1,2-benzoypyrene or less commonly, as o-hydroxycinnamic acid-8-lactone), and it has been well studied. The pharmacological and biochemical properties and therapeutic applications of simple coumarins depend upon the pattern of substitution (Hoult and Paya, 1996).

Several natural products with a coumarinic moiety have been reported to have multiple biological activities (Ivanovska et al., 1994; Paya et al., 1994; Chang and Chiang, 1995; Chang et al., 1996; Fylaktakidou et al., 2004). It is to be expected that coumarins might affect the formation and sca-
Antioxidative activity of fraxin

Fraxin (7-hydroxy-6-methoxycoumarin 8-glucoside), structurally a derivative of a coumarin glucoside, is a colorless crystalline substance (C_{16}H_{18}O_{10}) found in the bark of the ash (Fraxinus), and along with esculin in the bark of the horse-chestnut. It shows a delicate blue-green fluorescence in alkaline solutions; called also paviin and fraxoside. Fraxin shows its antioxidative effect through inhibition of cyclo AMP phosphodiesterase enzyme (Schempp et al., 2000). It also shows analgesic effect like a non-steroidal anti-inflammatory drugs (von Krueeneder et al., 1995; Klein-Galczinsky, 1999). Previous studies also suggested that fraxin isolated from some plants showed various activities. Fraxin from *Fraxinus excelsior* had anti-inflammatory and antimetastatic properties, the former probably because of its direct action on cells, predominantly on macrophages inhibitory effect on 5-HETE production (Ivanovska et al., 1994; Paya et al., 1995; Chang et al., 1996; Fylaktakidou et al., 2004). Coumarin derivatives have recently attracted much attention because of their broad pharmacological activities.

Fraxin was extracted from *Weigela florida var. glabra* (Caprifoliaceae), purified and characteri-

Materials and Methods

Chemicals

Fraxin was extracted from *Weigela florida var. glabra* leaves (Caprifoliaceae), purified and characteri-
ized as described (Morikawa et al., 2002). The methanolic extract was suspended with water and then partitioned with ether. The aqueous fraction was submitted to column chromatography on Diaion HP-20 with gradient solvent system as follows: H₂O, 20% MeOH, 60% MeOH, MeOH (Jeong et al., 1999). The 20% and 60% MeOH fractions were subjected to sephadex LH-20 column chromatography using 15%, 50% MeOH to yield compound. The structure of compound was elucidated by spectroscopic parameters of ¹H-NMR, ¹³C-NMR, FT-IR and FAB-MS, and identified as fraxin.

HUVEC culture and cell viability assay
HUVECs were obtained from full-term placenta after delivery. Patient consent was obtained from each individual and the use of tissue samples was approved by the ethics committee of our institution. HUVECs were isolated from umbilical cords. Veins were cannulated and flushed first with phosphate-buffered saline (PBS) (170 mM NaCl, 10 mM Na₂HPO₄, 3.3 mM KCl, and 1.8 mM KH₂PO₄, pH 7.4) before being filled with 0.2% (v/v) collagenase type II (Sigma, St. Louis, MO) in PBS. Following 10 min incubation at 37°C (5% CO₂), the collagenase was removed by flushing with M-199 medium, and cells were centrifuged for 10 min at 1000 g. Cells were then resuspended in growth medium (M-199) and seeded into 25-cm² cell cultured flask previously coated with 1% (v/v) liquid gelatin (Sigma) with PBS. HUVECs were grown to confluence before starting the treatments in 96-well micro plates in a final volume of 100 µl culture medium containing 10⁵ cells per well. After allowing the cultures to confluence, chemicals and H₂O₂ treatments were followed with certain intervals.

To determine the cell viability, XTT assay kit was used as described in company's manual (R&D Systems Inc., Minneapolis, MN). Two cases of oxidative stress were applied to pre-incubated HUVECs with chemicals for 1 h: the treatment with 1 mM H₂O₂ for 1 h and the treatment with 0.1 mM H₂O₂ for 48 h. Concentrations of fraxin were varied from 0 to 0.5 mM.

Analytical methods
CAT activity was determined using Oxis research kit (OXISResearch, Portland, OR). After addition of 10 mM H₂O₂ cell lysates were incubated for 1 min and mixed with chromogen substrate followed by addition of stopping reagent. Developed color for 10 min incubation was detected at 520 nm. The rate of change in absorbance was converted to units of enzyme activity, determined from a standard curve using CAT. Enzyme activity was then standardized to mg protein.

SOD enzymatic activity was determined using the assay described in Oxis research SOD-525 kit manual (OXISResearch, Portland, OR). In brief, cell lysates were treated with mercaptan eliminating reagent and chromogenic substrate of SOD was added to the samples. SOD activity was determined by measuring the absorbance at 525 nm over time.

The extent of lipid peroxidation was determined by using the assay described in Oxis research LPO-586 kit manual (OXISResearch, Portland, OR). In brief, the LPO-586 assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenyliindole with malondialdehyde (MDA) and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-hydroxylanlen reacts with 2 molecules of reagent, N-methyl-2-phenyliindole, to yield a stable chromophore with maximal absorbance at 586 nm.

Intracellular ROS levels were determined by ROS mediated conversion of non-fluorescent 2',7'-dichlorofluorescein diacetate (DCFH) to DCF. The study was performed by slight modification of a method described previously (Zapolska-Downar et al., 1999). Cells were cultured overnight in 6-well culture plates and preincubated with fraxin for 1 h. Next, 1 mM of H₂O₂ was added to the cells and the cells were incubated for 1 h. The cells were washed with M-199 media twice and followed by incubation with 0.02 mM DCFH for 30 min in the dark. After incubation, 3 times of washing and lysis of the cells with 0.1% Triton X-100 in 0.1 M Tris was carried out. Relative fluorescence intensity of DCF at an emission of 525 nm and an excitation of 475 nm of each sample was measured using Victor V² (Perkin-Elmer).

1,1-diphenyl-3-picrylhydrazyl (DPPH) radical has a deep violet color due to its unpaired electron and radical scavenging can be followed spectrophotometrically by the loss of absorbance at 517 nm as the pale yellow non-radical form is produced. 0.08 mM of DPPH solution in ethanol was prepared and added to various concentrations of fraxin in ethanol. Absorbance changes at 517 nm for 10 min with 10 sec of interval were monitored.

Assessment of gene expression profiles in fraxin or H₂O₂ treated HUVECs
For differential display reverse transcription-polymerase chain reaction (DDRT-PCR) of mRNA (Liang and Pardee, 1992), HUVECs were obtained from full-term placenta after delivery. Total RNA was extracted from HUVECs treated with or without 0.2 mM of fraxin for 8 h using a RNA extraction kit (RNeasy total RNA kit; Qiagen Inc., Valencia, CA) and 0.2 µg of total RNA was used to generate cDNA in a reverse transcription reaction (RNAimage™ kit, GenHunter, MA). With the use of the differential
display kit (RNAimage™ kit), we performed PCR using oligo-dT primers and arbitrary sequences, each 13 bases in length according to the manufacturer’s recommendations. After cDNAs of 3’ termini of mRNAs were generated, the PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. From the films, differentially expressed genes, which were overexpressed or down-regulated in fraxin or H₂O₂ treated HUVECs, were identified and were then subcloned into the pGEM-T easy vector with the use of the TA cloning system, and subjected to an automatic sequencing analysis.

Northern blot analysis
The extracted total RNA of the cells was treated with DNase I and reverse transcribed with random hexamer priming (Clontech). By using the product as template, PCR was performed by using 5’-GGA-TGCCCTAAATGAGACCA-3’ as the sense primer and 5’-GAGAGAAGGGCATCAAGCTG-3’ as the antisense primer (Clusterin; GenBank accession no. BC010514). This RT-PCR product size was 455 bp. This 455-bp product was used as a probe for Northern blot analysis. Northern blot analysis was carried out, in which 20 μg of denatured total RNA was electrophoresed on a 1.0% formaldehyde agarose gel and transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). Blot was hybridized with the randomly primed [³²P]-labeled 455-bp cDNA probe. Human β-actin cDNA control probe was used as a loading control.

Comparison of fraxin and chemicals structurally related with fraxin in cell viability enhancement
Based on structural similarity compared with fraxin, 18 chemicals showing more than 70% structural similarity were chosen among company’s chemical library system. Structural similarity was calculated by ChemFinder (Cambridgesoft, MA). Basically same experimental procedure with previous cell viability assay was used for fraxin-related chemical screening except that the concentration of each chemical and H₂O₂ was fixed at 0.02 mM and 0.2 mM, respectively. Chemicals were pretreated to HUVECs for 1 h and then cells were incubated with 0.2 mM H₂O₂ for 1 h. Less harsh condition (0.2 mM) than 1 mM of H₂O₂ was used to increase selection ratio in this primary screening, which would give more information about the relationship between chemical structures and activities. HUVECs’ survival was determined as about 60% at 0.2 mM H₂O₂ treatment. Experiments for each chemical were repeated three times and data were averaged. Viability recovery was calculated as relative recovery ratio, which means differences from cell viability of control. Control is viabilities of cells treated with H₂O₂ alone.

Results
Effects of H₂O₂ exposure on HUVECs in cell based assay
Cell based assay is basically to monitor the change of cell viability induced by oxidative stress, which was H₂O₂ exposure on HUVECs in our experiments. Preliminary experiments were performed to determine the effects of H₂O₂ exposure on HUVECs (Figure 1). The concentration of H₂O₂ was varied from 0.2 to 5 mM and cell death induced by H₂O₂ was monitored. Cell viability was also assessed after 0.5, 1 or 2 h. There were no significant differences in survival rate at each time point (data not shown). Cell viability was ranged from 70% (0.2 mM) to 10% (5 mM) and 40% of cells were viable at 1 mM H₂O₂ (Figure 1). Therefore, 1 mM H₂O₂ was used through all subsequent screenings to differentiate the effects of survival and death by chemicals.

Primary screening of fraxin using cell based assay
Fraxin was extracted, purified from Weigela florida var. glabbra and used as primary screening material to investigate whether fraxin is an effective antioxidative chemical, which may protect cells from oxidative stress. Since cell based assay is more direct assay to select antioxidative chemicals, it was employed as primary screening assay. Three different concentrations (0.02, 0.1, and 0.5 mM) of fraxin were treated for 1 h prior to the addition of 1 mM
H$_2$O$_2$ and relative cell viabilities in comparison to HUVECs alone were monitored (Figure 2A). In addition to high concentration of H$_2$O$_2$ treatment (1 mM), low concentration of H$_2$O$_2$ (0.1 mM) was introduced into HUVECs for 48 h to evaluate fraxin that may protect cells exposed to H$_2$O$_2$ at low concentration and for long time (Figure 2B). Fraxin improved cell survival rate more than 50% at 0.1 mM in cells exposed to H$_2$O$_2$ at high concentration (1 mM) and for short time (1 h) (about 40% cell survival rate) (Figure 2A). But in cells exposed to H$_2$O$_2$ at low concentration (0.1 mM) and for long time (48 h) (about 20% cell survival rate), fraxin improved cell survival rate more than 35% at 0.5 mM (Figure 2B).

**SOD and CAT assays**

In the present experiment, H$_2$O$_2$ exposure on HUVECs resulted in no significant change in either SOD or CAT activities in HUVECs and fraxin also did not affect activities of both enzymes in HUVECs (data not shown).

![Figure 2](image2.png)

*Figure 2.* Primary screening of fraxin using cell based assay. Cell viability assays were performed with three different concentrations of fraxin. Prior to incubation with H$_2$O$_2$, HUVECs were treated with 0.02 (opened bar), 0.1 (grey bar) and 0.5 (black bar) mM of fraxin for 1 h, respectively and then followed by 1 h incubation in 1 mM H$_2$O$_2$ (A) and 48 h incubation in 0.1 mM H$_2$O$_2$ (B). Blank denotes untreated control HUVECs.

![Figure 3](image3.png)

*Figure 3.* Intracellular LPO and ROS production. (A) LPO production of HUVECs with the treatment with H$_2$O$_2$ and/or fraxin. (B) Intracellular ROS production detected by DCF with the treatment with H$_2$O$_2$ and/or fraxin. Each value is the means ± SD of three independent experiments.
LPO assay and production of ROS

MDA level obtained from fraxin treatment on HUVECs alone was similar to the level in the HUVEC cultures without oxidative stress (Figure 3A). When the cells are treated with H₂O₂, however, there was a significant increase of MDA production to about 210%. This elevated MDA level was decreased to about 190% with treatment of fraxin prior to the addition of H₂O₂ (Figure 3A). Treatment with fraxin showed a protector effect with about 20% decrease in MDA production induced by H₂O₂.

To address the possibility that the increased cell viability in the presence of phytochemicals is due to decreased production of ROS inside cells, we measured intracellular concentrations of H₂O₂ in HUVECs. The effect of fraxin on cellular oxidation was determined by DCF fluorescence. HUVECs activated by H₂O₂ showed an increase in free radical level by about 60% over non-treated HUVECs (Figure 3B). Pretreatment with fraxin prior to the addition of H₂O₂ decreased intracellular H₂O₂ levels to about 39% (Figure 3B). Since fraxin showed high ROS scavenging effect, direct scavenging of ROS by fraxin could account for reduced intracellular H₂O₂.

Free radical scavenging activity

Fraxin exposure on HUVECs showed very high free radical scavenging activity at high concentration (Figure 4). At 0.5 mM, about 50% of free radicals were quenched. But free radical scavenging ability of fraxin was not efficient at used concentrations (0.02 mM).

Differential display and northern blot analysis

Using the DDRT-PCR, genomic expression levels in HUVECs were estimated after H₂O₂ exposure or fraxin treatment, respectively. Differentially expressed mRNAs by fraxin or H₂O₂ treatment were selected and compared to each other. Among identified genes, the change of gene expression pattern by fraxin in comparison to untreated HUVECs was interesting and unique. Fraxin upregulated anti-apoptotic genes (clusterin and apoptosis inhibitor 5) and tumor suppressor gene (ST13) (Table 1). Among three identified genes, clusterin, apoptosis inhibitor 5 and ST13 putative tumor suppressor, by

![Figure 4](image.png)

Figure 4. Free radical scavenging activity of fraxin. Free radical scavenging ability of fraxin determined by using DPPH assay. Eighty μM of DPPH solution in ethanol was prepared and added to various concentrations of chemical (0.02, 0.1 and 0.5 mM) in ethanol. Absorbance changes at 517 nm for 10 min with 10 s of interval were monitored. Each value is the means ± SD of three independent experiments.

![Figure 5](image.png)

Figure 5. Clusterin gene expressions in HUVECs. Northern blot analysis was performed to determine the expression pattern in HUVECs. Blot was hybridized with the randomly primed [³²P]-labeled 455-bp cDNA probe for clusterin. Human β-actin cDNA probe was used as a loading control.

| Expression level | Gene                                      |
|------------------|-------------------------------------------|
| Control          | Strong, SGP-2, Clusterin (anti-apoptotic activity) |
| Medium           | Strong                                   |
| Weak             | Medium                                   |
| Weak             | Strong                                   |

Table 1. Patterns of differentially expressed genes by fraxin or H₂O₂.
using the DDRT-PCR, clusterin showed the most strong upregulation after treatment with fraxin compared with apoptosis inhibitor 5 or ST13 putative tumor suppressor (Table 1). Accordingly we performed Northern blot analysis to reconfirm the upregulation of clusterin in fraxin-treated HUVECs. As expected, clusterin was upregulated in fraxin-treated HUVECs compared to control HUVECs or H$_2$O$_2$-treated HUVECs (Figure 5).

**Cell viability assay with chemicals structurally related to fraxin**

Based on structural similarity compared with fraxin, 18 chemicals showing more than 70% structural similarity were chosen and previous cell viability assay was used for fraxin-related chemical screening (Table 2). Through viability assay, seven chemicals related to fraxin showed more than 18% (two-fold) enhancement of viability in comparison with control (Table 2 and Figure 6). Seven chemicals related to fraxin were fraxidin methyl ether (chemical No. 1, 29.4% enhancement of viability), prenyletin (chemical No. 2, 26.4%), methoxsalen (chemical No. 3, 20.8%), diffratic acid (chemical No. 4, 19.9%), rutoside (chemical No. 5, 19.1%), xanthyletin (chemical No. 6, 18.4%), and kuhlmannin (chemical No. 7, 18.2%) (Figure 6). Percentages in parenthesis

| No. | Structure | Name                | Survival (%) | No. | Structure | Name                | Survival (%) |
|-----|-----------|---------------------|--------------|-----|-----------|---------------------|--------------|
| 1   |           | FRAXIDIN METHYL ETHER | 29.4         | 11  |           | SCOPOLETIN          | 15.5         |
| 2   |           | PRENYLETIN          | 26.4         | 12  |           | DIOSMIN             | 11.9         |
| 3   |           | METHOXSALEN         | 20.8         | 13  |           | HERNIARIN           | 9.2          |
| 4   |           | DIFFRATIC ACID      | 19.9         | 14  |           | CITROPTEN           | 9.0          |
| 5   |           | RUTOSIDE            | 19.1         | 15  |           | DIHYDRO-OB莉QUIN    | 7.5          |
| 6   |           | XANTHYLETIN         | 18.4         | 16  |           | CITROPTEN           | 5.0          |
| 7   |           | KUHLMANNIN          | 18.2         | 17  |           | DICUMAROL           | -9.2         |
| 8   |           | WARFARIN            | 17.8         | 18  |           | BERGAPTENE          | -92.7        |
| 9   |           | QUERCITRIN          | 17.0         | FX  |           | Fraxin              | 9.3          |
| 10  |           | AESCULIN            | 16.6         |     |           |                     |              |

*Survival: relative survival ratio (%) to control
Antioxidative activity of fraxin

Fraxin methyl ether showed the highest activity among tested 18 chemicals. These 7 chemicals were selected as primary hits since those were considered as chemicals showing significant enhancement of viability in comparison with fraxin, which showed only 9.3% enhancement of cell viability (Table 2 and Figure 7).

Figure 6. Cell viability recovery of 18 structurally fraxin-related chemicals. Filled bars indicate fraxin-related chemicals showing cell viability over 18% and opened bars indicate fraxin-related chemicals showing cell viability under 18%. Experiments for each chemical were repeated three times and data were averaged. Viability recovery was calculated as relative recovery ratio, which means differences from cell viability of control. Control denotes viability of cells treated with H_2O_2 alone.

Figure 7. The chemical structure of fraxin and seven chemicals, fraxidin methyl ether, prenylalin, methoxsalen, difftratic acid, rutoside, xanthyletin and kuhlmannin.
Discussion

Reducing reactive oxygen species in the body is one of main efforts towards protection of various diseases such as aging process, cancer, diabetes and neurodegenerative diseases. Many natural products including flavonoids, coumarins, polyols have been studied for the characterization and the development as antioxidative reagents (Finkel and Holbrook, 2000; Han, 2003; Whang et al., 2005).

Coumarins comprise a group of phenolic compounds widely distributed in natural plants (Egan et al., 1990; Kaneko et al., 2003), and they have recently attracted much attention because of their broad pharmacological activities. Among these compounds, esculetin (6,7-dihydroxycoumarin) shows scavenging activity against ROS such as superoxide radicals (Chang et al., 1996) and hydroxyl radicals (Hiramoto et al., 1996), and inhibits lipid peroxidation in rat livers (Martin-Aragon et al., 1998). But fraxin (7-hydroxy-6-methoxycoumarin 8-glucoside), a coumarin derivative, was shown neither to inhibit lipid peroxidation nor to scavenge superoxide radicals or hypochlorous acid to any significant extent, although fraxin was capable of rapidly reacting with hydroxyl radicals, and fraxin scavenged alk peroxy radicals with useful potency (Hoult and Paya, 1996). Inconsistent with previous observations (Hoult and Paya, 1996), cell based assay used in this study to monitor the change of cell viability induced by oxidative stress revealed that fraxin enhanced the viability of HUVECs.

In our studies, fraxin showed protective effects against H2O2-mediated oxidative stress. Recovering the viability of damaged HUVECs and reducing the LPO and the internal ROS level by fraxin clearly explains the protective effect against oxidative stress. Although free radical scavenging ability of fraxin was not efficient at used concentration (0.02 mM), about 50% of free radicals were quenched at high concentration (0.5 mM). While fraxin did not affect the biological activities involved in antioxidative mechanisms such as catalase and SOD in the biological systems, induced lipid peroxidation and internal ROS level by H2O2 in the HUVECs were reduced by fraxin treatment. From these results, direct deactivation of ROS probably is primary reason for the protective effect of fraxin from H2O2-mediated oxidative stress.

To find out more efficient antioxidants from the fraxin derivatives, 18 chemicals showing more than 70% structural similarity with fraxin were chosen and cell viability assay was used for fraxin-related chemical screening. Through viability assay, seven chemicals related to fraxin showed more than 18% enhancement of viability in comparison with control. Seven chemicals related to fraxin were fraxidin methyl ether, prenylmetin, methoxsalen, difieric acid, rutoside, xanthyletin and kuhlmannin. Fraxidin methyl ether showed the highest enhancement of cell viability (29.4%) among tested 18 chemicals. Fraxidin with a coumarinic moiety has been reported to have several beneficial properties, including antioxidant, anti-inflammatory, and anti-diabetogenic effects (Kimura et al., 1985; Kim et al., 1999; Fort et al., 2000). In polymorphonuclear leukocytes, fraxidin inhibited the formation of the cyclooxygenase product, hydroxy-5,8,10-heptadecatrienoic acid (HHT), strongly by inhibition of the formation of 5-hydroxy-6,8,11,14-eicosatetraenoic acid from arachidonic acid (Kimura et al., 1985). It is to be expected that fraxidin can reduce tissue edema and inflammation. Moreover fraxidin inhibited the formation of inducible nitric oxide synthase (Kim et al., 1999) and showed antihyperglycemic activity (Fort et al., 2000). These 7 chemicals including fraxidine were selected as primary hits since those were considered as chemicals showing significant enhancement of viability in comparison with fraxin. Further investigation would be necessary to optimize its activity and drug-likeness for the development as a drug candidate.

When the perturbation of cells by exogenous oxidants such as H2O2 is severe, cells may respond in two different ways, survival or insult by undergoing cell death. In cellular systems, a number of stress response mechanisms help cells adopt damage or resist to the stress. In general, the heat shock response and the ERK, PI3K/Akt and NF-kB signaling pathways exert a pro-survival influence against oxidative damages. In contrast, activation of p53, JNK and p38 are linked to apoptosis, which leads damaged cells to the death to remove from the multi-cellular systems (Finkel and Holbrook, 2000).

To link the evidences of antioxidative ability of fraxin to molecular mechanisms, the analysis of gene expression patterns by differential display method was carried out. Our results suggest alternative mechanisms for the antioxidative effects of fraxin. Differential display RT-PCR revealed that fraxin upregulated anti-apoptotic genes (clusterin and apoptosis inhibitor 5) and tumor suppressor gene (ST13). This finding is interesting that fraxin induced anti-apoptotic genes (Wolf and Green, 2002; Thiede and Rudel, 2004) because apoptosis and aging share common mechanisms in oxidative stress and mitochondrial involvement (Lenaz et al., 1998). Cells in the brain deploy multiple mechanisms to maintain the integrity of nerve cell circuits, and to facilitate responses to environmental demands and promote recovery of function after injury (McGeer et al., 1992). The mechanisms include production of neurotrophic factors and cytokines, expression of
various cell survival-promoting proteins (e.g., protein chaperones, antioxidant enzymes, Bcl-2 and inhibitor of apoptosis proteins), protection of the genome by telomerase and DNA repair proteins, and mobilization of neural stem cells to replace damaged neurons and glia (McGeer et al., 1992).

Clusterin, a secreted mammalian chaperone, is upregulated in response to complement membrane attack complex formation in Alzheimer disease (Koch-Brandt and Morgans, 1996; Wilson and Easterbrook-Smith, 2000). Clusterin is a cell survival gene, exerting a protective function on the surviving bystander cells. Based on these results that clusterin and apoptosis inhibitor 5 regulate apoptosis and survival signaling pathways for the cell survival, cellular protective ability of fraxin against oxidative stress may be linked to the induction of clusterin and apoptosis inhibitor 5 genes by fraxin. These results suggest that fraxin-induced genes may play roles in cellular protective functions of fraxin. Further investigation with identified genes is intensively undergoing to support this preliminary linkage between antioxidative activities and molecular mechanisms involved in fraxin-mediated cell survival.

This study suggests that fraxin and fraxin-related chemicals protect HUVECs from oxidative stress. In view of the established low toxicity, relative cheapness, presence in the diet and occurrence in various normal human herbal remedies of coumarins, it appears prudent to evaluate their properties and applications further. Further studies are needed in various normal human cells including immune cells regarding its antioxidative and neo-vascularization effects.

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