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), prenyletin (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 -pyrone 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-benzopyrone 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-

vening of ROS and influence processes involving free radical-mediated injury. Coumarin can reduce tissue edema and inflammation. Moreover coumarin and its 7-hydroxy-derivative inhibit prostaglandin biosynthesis, which involves fatty acid hydroperoxy intermediates. Natural products like esculetin, fraxetin, daphnetin and other related coumarin derivatives are recognised as inhibitors not only of the lipoxygenase and cyclooxygenase enzymic systems, but also of the neutrophil-dependent superoxide anion generation (Ivanovska *et al.*, 1994; Paya *et al.*, 1994; Chang and Chiang, 1995; Chang *et al.*, 1996; Fylaktakidou *et al.*, 2004). Coumarin derivatives have recently attracted much attention because of their broad pharmacological activities.

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 Krüedener *et al.*, 1995; Klein-Galczynsky, 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). And the flowering or manna, ash, *Fraxinus ornus* L. [Fam. Oleaceae], the bark contains hydroxycoumarins, secoiridoid glucosides, phenylethanoids and flavonoids. Biological studies of hydroxycoumarins reveals significant antimicrobial, anti-inflammatory, immunomodulatory, antiviral and diuretic activities that support the use of this bark in folk medicine (Guarrera, 1999; Kostova, 2001). These results show that the traditional use of *Fraxinus ornus* stem bark in the treatment of inflammatory disorders is at least partially due to its coumarin constituents. Fraxin is also known to act as a choleric agent for stimulating bile flow and aiding digestion, and has noted activity for preventing the development of abnormal growths (Iossifova *et al.*, 1994). In the study of effect of some hydroxycoumarins on complement-mediated hemolysis in human serum, the interactions between esculin, esculetin, fraxin, fraxetin, as well as their acetylated and methylated derivatives and non-cell system participating in inflammatory processes, comprised of serum complement proteins, were investigated *in vitro*. Fraxin was able to enhance hemolysis (Ste-

fanova *et al.*, 1995). In this study, as the constituent of the leaves of *Weigela florida* var. *glabra* (Caprifoliaceae), fraxin was studied phytochemically because the leaves of *Weigela florida* var. *glabra* have not been studied as medicinal plant.

ROS and reactive nitrogen species (RNS) constantly generated in normal condition by aerobic metabolism include free radicals such as superoxide anion, hydroxyl radicals, nonradical hydrogen peroxide, peroxynitrite, nitroxyl anion and nitric oxide (Beckman and Ames, 1998; Curtin *et al.*, 2002; Droge, 2002). In normal biological systems, redox homeostasis is maintained by controlling the balance between ROS production and various types of scavengers called antioxidants. Transient changes in oxidant-antioxidant balance are normally regulated by changing the production of counter species and reached to the steady-state over time (Shull *et al.*, 1991; Nakamura *et al.*, 1994; Adler *et al.*, 1999; Zhang and Storz, 2000; Droge, 2002). The persistent production of abnormally large amount of ROS or RNS, however, may lead to persistent changes in signal transduction and gene expression, which in turn may give rise to certain diseases.

Various enzymatic and non-enzymatic self defense systems against oxidative stress including transiently over-produced ROS inside cells or exposure to external ROS inducing species have been identified and studied extensively. Catalase (CAT), SOD, and glutathione peroxidase (GPX) are the examples of enzymatic defense systems and non-enzymatic systems include tocopherol, ascorbate, urate and glutathione (Sies, 1993).

The endothelium is known to be sensitive to injury caused by ROS (Valen *et al.*, 1999), and in contrast, free radicals released by endothelial cells mediate the oxidation of low density lipid (LDL) (Zapolska-Downar *et al.*, 1999). HUVECs have been used to study oxidative stress related researches and oxidative stress mediated by H_2O_2 damages cellular functions of HUVECs via various mechanisms (Valen *et al.*, 1999; Estrada-Garcia *et al.*, 2002; Waxman *et al.*, 2003).

In the present study, we investigated cellular protective activity of coumarin compound, fraxin (7-hydroxy-6-methoxycoumarin 8-glucoside), under oxidative stress, identified genes expressed differentially by fraxine treatment and compared antioxidant effect of fraxin with its structurally related chemicals.

Materials and Methods

Chemicals

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

zed 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 solvents 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 culture 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 10⁴ cells per well. After allowing the cultures to grow 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 (OXIS Research, 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 (OXIS Research, 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 (OXIS Research, Portland, OR). In brief, the LPO-586 assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenyl indole with malondialdehyde (MDA) and 4-hydroxyalkenals at 45 °C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of reagent, N-methyl-2-phenyl indole, 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 V³ (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 (RNA ImageTM 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. Con-

Figure 1. Cell viability dependent on the concentration of H₂O₂. Various concentrations of hydrogen peroxide (0–5 mM) were treated into HUVEC culture media and cell viability was assessed. 0.5 mM of H₂O₂ induced about 50% cell death and 1 mM of H₂O₂ resulted in about 60% cell death. Each value is the means ± SD of three independent experiments.

trol 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. *glaberrima* 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

H₂O₂ and relative cell viabilities in comparison to HUVECs alone were monitored (Figure 2A). In addition to high concentration of H₂O₂ treatment (1 mM), low concentration of H₂O₂ (0.1 mM) was introduced into HUVECs for 48 h to evaluate fraxin that may protect cells exposed to H₂O₂ 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 H₂O₂ at high concentration (1 mM) and for short time (1 h) (about 40% cell survival rate) (Figure 2A). But in cells exposed to H₂O₂ 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, H₂O₂ 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. Primary screening of fraxin using cell based assay. Cell viability assays were performed with three different concentrations of fraxin. Prior to incubation with H₂O₂, 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 H₂O₂ (A) and 48 h incubation in 0.1 mM H₂O₂ (B). Blank denotes untreated control HUVECs.

Figure 3. Intracellular LPO and ROS production. (A) LPO production of HUVECs with the treatment with H₂O₂ and/or fraxin. (B) Intracellular ROS production detected by DCF with the treatment with H₂O₂ and/or fraxin. Each value is the means $\pm$ 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 H_2O_2 , 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 H_2O_2 (Figure 3A). Treatment with fraxin showed a protector effect with about 20% decrease in MDA production induced by H_2O_2 .

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 H_2O_2 in HUVECs. The effect of fraxin on cellular oxidation was determined by DCF fluorescence. HUVECs activated by H_2O_2 showed an increase in free radical level by about 60% over non-treated HUVECs (Figure 3B). Pretreatment with fraxin prior to the addition of H_2O_2 decreased intracellular H_2O_2 levels to about 39% (Figure 3B). Since fraxin showed high ROS scavenging effect, direct scavenging of ROS

by fraxin could account for reduced intracellular H_2O_2 .

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 H_2O_2 exposure or fraxin treatment, respectively. Differentially expressed mRNAs by fraxin or H_2O_2 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. Free radical scavenging activity of fraxin. Free radical scavenging ability of fraxin determined by using DPPH assay. Eighty μ M 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 $\pm$ SD of three independent experiments.

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 [32 P]-labeled 455-bp cDNA probe for clusterin. Human β -actin cDNA probe was used as a loading control.

Table 1. Patterns of differentially expressed genes by fraxin or H_2O_2 .

Expression level			Gene
Control	Fraxin	H_2O_2	
Medium	Strong	Weak	SP40, SGP-2, Clusterin (anti-apoptotic activity)
Weak	Medium	Weak	Apoptosis inhibitor 5
Weak	Strong	Weak	HSP interacting protein (Hip). ST13 Putative tumor suppressor

using the DDRT-PCR, clusterin showed the most strong upregulation after treatment with fraxin compared with apoptosis inhibitor 5 or ST13 putative tumorsuppressor (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_2O_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 methylether (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

Table 2. Screening results of fraxin-related chemical library. ^a

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-OBLIQUIN	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				

^aSurvival: relative survival ratio (%) to control

Figure 6. Cell viability recovery of 18 structurally fraxin-related chemicals. Filled bars indicate fraxin-related chemicals showing cell viability over 18%, and open 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 cell treated with H_2O_2 alone.

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

indicate the relative viability enhancement. Fraxidin methyl ether showed the highest activity among tested 18 chemicals. These 7 chemicals were selected as primary hits since those were considered as che-

micals showing significant enhancement of viability in comparison with fraxin, which showed only 9.3% enhancement of cell viability (Table 2 and Figure 7).

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 antioxidant 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 alkylperoxy 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 H_2O_2 -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 H_2O_2 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 H_2O_2 -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, prenyletin, methoxsalen, diffratic 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 H_2O_2 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- κ B 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 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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