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Anti-apoptotic Effect of Steam Exploded *Quercus variabilis*[1]

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ABSTRACT

We hypothesized that the extract from steam exploded *Q. variabilis* might be cytoprotective for tenofibroblasts cells during oxidative stress. In the present study, the extracts obtained from steam exploded (severity log *Ro* 4.68) *Q. variabilis* contained high quantities of phenolics and flavonoids contents. Also, the extracts appear to have, on these tenofibroblasts, a protective effect against oxidative stress. Tenofibroblasts cells incubated with the extracts and stressed with H_2O_2 showed an increase in cell viability by MTT assay. The extracts is found to inhibit H_2O_2-induced apoptosis in tenofibroblasts cells, as shown by Annexin V/PI double staining analysis. Western blot data showed that in the extracts/H_2O_2-treated cells, the extracts inhibited the H_2O_2-dependent phosphorylation of ERK and p38. From these results, it is suggested that the extracts showed the protective effect on H_2O_2-mediated oxidative stress. The main chemical compounds of the extract was identified as 1,8-cineole by GC-MS analysis. The anti-apoptosis activity is accordingly believed to be attributable to the 1,8-cineole.

Keywords : anti-apoptotic, anti-oxidant, *Quercus variabilis*, steam explosion, achilles tenofibroblasts

1. INTRODUCTION

Quercus species are well known for their polyphenolic contents and traditional usage in worldwide (Şöhretoğlu *et al*. 2012). The species are rich source of saponins and phenolics, particularly tannins, flavonoids and proanthocyanidins (Romussi *et al*. 1994; Şöhretoğlu and Sakar 2004). *Quercus* species have been shown to possess antimicrobial, antiinflammatory, gastroprotective, antioxidant, antitumoural properties (Şöhretoğlu *et al*. 2007). *Quercus* species are used as antifungal, antidiarrheic, astringent, for the treatment of hemorrhoid, tonsillitis, inflammation of oral and anal mucosa. Moreover, the decoction of these plants is used for burns

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and added to ointments for wound healing (Sezik *et al*. 2001; Gurhan and Ezer 2004).

Recently, the steam explosion treatment has been attractive for the degradation and separation of structural components, i.e. cellulose, hemicellulose, and lignin. The principle of the steam explosion treatment is the steam hydrolysis at high temperature and pressure, followed by sudden reduction of the pressure for physical treatment of the hydrolyzed product to produce low-molecular weight substances. The steam explosion process has been tried and found to be useful in the low cost biomass conversion of wood and crop residues (Taylor and Yu 1995). It has been found as an effective pretreatment of wood biomass for biotechnological conversion for production of food, feed and chemicals. The steam explosion treatment results in substantial breakdown of the lignocellulosic structure, hydrolysis of the hemicellulosic fraction, depolymerization of the lignin components and defibration. Due to the disruption of the cell-wall matrix, the steam explosion treatment has been employed as an effective pretreatment process for extracting and separating bioactive phytochemicals from plant tissues (Jackobsons *et al*. 1995; Kallavus and Cravitis 1995).

Reactive Oxygen Species (ROS) can cause cell death via apoptosis (Wood and Youle 1994; Jacobson 1996). Excessive ROS formation can induce oxidative stress, leading to cell damage that can culminate in cell death. Therefore, cells have antioxidant networks to scavenge excessively produced ROS. Hydrogen peroxide (H_2O_2), one of main ROSs, exerts its toxic effects mainly through forming the highly reactive hydroxyradical in the presence of the ferrous ion (Fenton 1984).

The achilles tendon, well known as the calcaneal tendon or the tendo calcanei, is a tendon of the posterior leg. It serves to attach the plantaris, gastrocnemius (calf) and soleus muscles to the calcaneus (heel) bone. In human beings, the tendon passes posterior to the ankle. It is the thickest and strongest tendon in the body. Apoptosis is important in the development of tendon degeneration in rotator cuff, patellar and achilles tendons (Chen *et al*. 2010; Scott *et al*. 2005; Skutek *et al*. 2003; Tuoheti *et al*. 2005; Yuan *et al*. 2002). Apoptosis is a series of biochemical events, including blebbing, loss of membrane asymmetry, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation, all of which eventually lead to the termination of the cell (Kerr *et al*. 1972; Chen *et al*. 2010).

The activity *in vitro* of the extracts from steam exploded wood was demonstrated antioxidant activity (Ando *et al*. 1986; Cruz *et al*. 1999; Eulogio Castro *et al*. 2008; Enma Conde *et al*. 2009; Larsson *et al*. 1999). However, the extracts are few published report (Nam *et al*. 2014) in the literature about the anti-apoptotic activity.

In the present study, this work is to evaluate the effect of steam explosion condition (severity log *Ro*) of *Q. variabilis* on the phenolic compound content and flavonoid content of the ethanol extract. Also, the possible protective effects of the ethanol extract against cytotoxicity

Table 1. Steam explosion experimental design

Reaction temperature (℃)	Reaction time (min)	Severity log (Ro)
225	5	4.38
225	10	4.68
225	20	4.98
225	30	5.15

induced by hydrogen peroxide (H_2O_2) on rat achilles tenofibrloblasts cells were investigated using MTT assay, flow cytometry, ROS and western blot analysis, respectively.

2. MATERIALS and METHODS

2.1. Raw material

Quercus variabilis was collected at Gyeongsang National University Research Forest, Sancheong, Korea and then air-dried and chopped to 2～3 cm.

2.2. Preparation of extract

2.2.1. Steam explosion

Steam explosion pretreatment, which was used as a physicochemical pretreatment, was carried out in a batch pilot unit equipped with a 1L reaction vessel. The "severity parameter" (*Ro*) was used to map the destruction, desegregation, and depolymerization of *Q. variabilis*. *Ro* was calculated using the following relation :

$$Ro = \{t * \exp[(T - 100)/14.5]\} \cdots\cdots\cdots\cdots (1)$$

where T is temperature (℃) and t time (min). A steam temperature of 225℃ and pretreatment time of 5, 10, 20 and 30 min were applied to realize a severity parameter value (Table 1). After the saturated steam exposure, a ball valve at the bottom of the reactor was opened suddenly to bring the reactor rapidly to atmospheric pressure. The steam exploded material was recovered in a cyclone and after cooling to about 40℃ filtered for solid recovery. The solid fraction was extracted.

2.2.2. Extraction

The solid fraction was extracted at room temperature in 95% (v/v) ethanol for 24 hours. The extract was then filtered and concentrated with a vacuum rotary evaporator (Rotavapor R-3, Buchi, Switzerland) under low pressure. Then the filtrate was freeze-dried in a freezing-dryer (TFD Series, Ilsin, Korea) and stored at -70℃. The freeze-dried obtained was dissolved in dimethyl sulfoxide (DMSO) to give a final extract concentration ranging from 1.25, 2.5, 5 and 10 ug/mℓ.

2.3. Quantitive analysis of phenolics and flavonoids content

The phenolic content was determined using Folin-Ciocalteu reagent. The extract was separately mixed with 5 mℓ of Folin-Ciocalteu

reagent. Sodium carbonate (7.5 %, 4 mℓ) was added to the mixture, shaken for a few seconds, and then incubated for 30 min at 40℃. The absorbance was read at 765 nm. The phenolic compound content was expressed in mg/g gallic acid equivalent.

The flavonoid content was determined by the spectrophotometric method. A mixture consisting of 0.5 mℓ 2 % aluminium chloride ($AlCl_3$) in ethanol and 0.5 mℓ of extract was prepared. It was left for 60 min at room temperature and the absorbance measured at 420 nm. The flavonoid content was expressed as *mg/mℓ* equivalent to quercetin.

2.4. Primary culture of rat achilles tendon cells

Achilles tendons from 6 weeks Sprague-Dawley rats (weighing 200-250 g) were excised. The excised tendon was washed twice in phosphate-buffered saline (PBS). Each tendon was then cut into small pieces of approximately 1.5-2.0 mm (six pieces in total) and these pieces were individually placed in six-well culture plates. After 5 minutes of air-drying for better adherence 0.5 mℓ of DMEM (Dulbecco's modified Eagle's medium), with 30% fetal bovine serum (FBS) (FBS, Gibco, USA). 100 IU/mℓ penicillin, and 100 mg/mℓ streptomycin in a humidified 5% CO_2 atmosphere at 37℃. After 2 weeks, the cells reached 90% confluence. The cells were then trypsinized for 5 minutes, centrifuged for 5 minutes at 1,500 rpm, and expanded in a second passage. The cells were then harvested with trypsin and cryopreserved. These cryopreserved third-passage cells were then thawed and used for all experiments in the current study.

2.5. MTT assay for cell viability

Tenofibroblasts cells were plated at a density of 5×10^4 cells/well in 24-well plates and the cell viability were measured using the Microculture tetrazolium (MTT) assay (Hansen *et al*. 1989). Oak extract was added to the wells (1.25, 2.5, 5 and 10 ug/mℓ) and incubated for 24 hours before the addition of hydrogen peroxide (H_2O_2, final concentration 0.5 mM). Briefly, at the indicated time after the treatment, 50 ug/mℓ MTT was added to 1 mℓ medium and incubation continued at 37℃ for 1 hour. MTT was removed and the colored formation was dissolved in dimethyl sulfoxide (DMSO). Absorbance at 570 nm was determined and used for the measurement of the proportion of surviving cells. The viability of tenofibroblasts cells in each well was presented as percentage of control cells.

2.6. Flow cytometry analysis

Apoptotic cells were quantified by Annexin V/PI double staining. Annexin V has a high affinity for phosphatidylserine which is translocated from the internal to the external surfaces of the cell membrane when tenofibroblasts cells undergo apoptosis. In this study, the cells were harvested after the treatment by tenofibro-

blasts cells and oak extract for different dose (1.25, 2.5, 5 and 10 ug/mℓ), and then washed twice with PBS, and the concentration was adjusted to 1×10^6 cells/mℓ. The pellets were resuspended in 70% ice cold ethanol and fixed at 4℃ for 24 hours. The cells were then centrifuged, and ethanol was removed by washing thoroughly with PBS. The cell pellets were resuspended in 1 mℓ binding buffer (10 mM Hepes/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) and incubated for 15 min. in the dark with Annexin V-FITC (20 ug/mℓ) and PI (50 ug/mℓ) at 4℃. Fluorescence was analyzed with an flow cytometry.

2.7. Reactive oxygen species (ROS) measurement

Production of intracellular ROS was detected by flow cytometry using DCF-DA (Bass *et al*. 1983). The tenofibroblasts cells were cultured in 60-mm tissue-culture dishes. The culture medium was replaced with new medium when the cells were 80% confluent. Following drugs incubation, the cells were treated with 5 mM DCF-DA for 10 minutes in the dark, washed once with PBS. To quantify the production rates of ROS, the cells were stained with DCF for 10 minutes, removed from the plate with trypsin (Trypsin, Gibco, USA), and collected on the FACs Calibur. Data were analyzed using Cell Quest software.

2.8. Western blot analysis

Tenofibroblasts cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer) containing protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem, USA) and centrifuged at 13,000 rpm at 4℃ for 15 minutes. Equal amounts of protein (30 ug) was separated with sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred onto a nitrocellulose membrane. After blocking with 5% skim milk, membranes were probed with primary anti-bodies against p-ERK, p-JNK, p-p38 overnight at 4℃. Peroxidase-conjugate anti-rabbit IgG were used for enhanced chemiluminescence (ECL) detection (ECL detection, Amersham Pharmacia Biotech, USA).

2.9. GC-MS analysis

The analysis of the extract was identified using an Agilent 7890A GC that was directly interfaced with an Agilent 5975C mass selective detector (MSD) (Agilent, Santa Clara, CA). An Agilent DB-5MS capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness) was used for the GC separation. Helium was used as the carrier gas at a flow rate of 0.8 mℓ min. The employed temperature program had the following settings: An initial temperature of 40℃ was held for 5 min and then increased to 250℃ at 5℃/min; this temperature was held for 10 min. The total GC runtime was 58 min. The temperatures of the interface, ion source, and quadruple were 280, 230, and

Table 2. Phenolic and flavonoid content of the extract from *Quercus variabilis* by steam explosion condition

Steam explosion condition (Severity log *Ro*)	Phenolic content (mg/g extract powder) [1)]	Flavonoid content (mg/g extract powder) [2)]
Control [3)]	36.8 ± 0.2 c [4)]	77.7 ± 4.5 d
4.38	41.5 ± 0.2 b	92.9 ± 0.7 c
4.68	69.1 ± 0.3 a	157.7 ± 1.1 a
4.98	39.0 ± 0.3 bc	147.1 ± 4.2 b
5.15	38.9 ± 0.1 bc	144.5 ± 3.0 b

[1)] Gallic acid equivalents
[2)] Quercetin equivalents
[3)] Untreated *Quercus variabilis*
[4)] Values with different alphabets in the same column are significantly different at $p<0.05$.

150℃, respectively. The volatile compounds were determined by comparing the MS fragmentation pattern to those of the standards and mass spectrum of the unknown peaks to those stored in the National Institute of Standards and Technology (NIST) GC-MS library, with the retention time of the standards obtained under the same conditions.

2.10. Statistical analysis

All experiments were triplicated, with the results expressed in each case as the mean standard deviation (SD) of the triplicate cultures. All statistical analyses were also performed via oneway ANOVA, followed by Tukey's multiple-comparison tests.

3. RESULTS and DISCUSSION

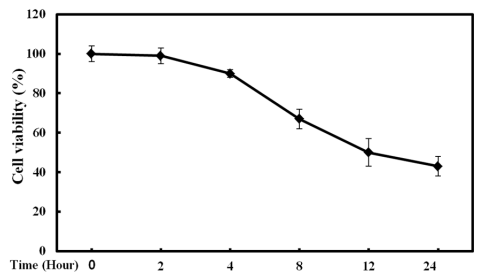
3.1. Effect of steam explosion condition on phenolic and flavonoid content

Table 2 shows the influence of steam explosion condition in the range severity log *Ro* 4.38 -5.15 on the phenolic and flavonoid content. The phenolic and flavonoid content of the extracts varies according to the steam explosion condition.

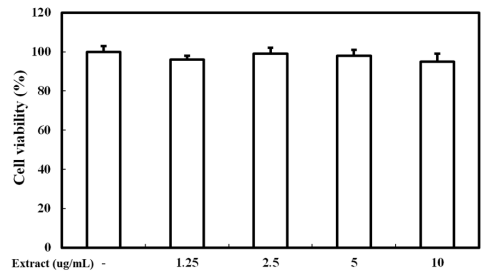
The phenolic content of the extracts from severity log *Ro* 4.38, severity log *Ro* 4.68, severity log *Ro* 4.98 and severity log *Ro* 5.15 was 41.5, 69.1, 39.0 and 38.9 mg/g, respectively. Maximum amount of phenolic content was obtained at severity log *Ro* 4.68 corresponded to 69.1 mg/g. The phenolic compound in the extract of severity log *Ro* 4.68 was increased by about 2 times compare with that of the untreated *Q. variabilis*.

The phenolic content of the extracts from severity log *Ro* 4.38, severity log *Ro* 4.68, severity log *Ro* 4.98 and severity log *Ro* 5.15 was 92.9, 157.7, 147.1 and 144.5 mg/g, respectively. Flavonoid content also varied according to the steam explosion condition.

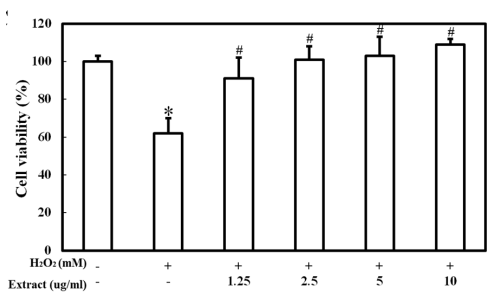
The extracts contained high quantities of phenolic and flavonoid content. Depolymerization of lignin during steam explosion increases its extractability by various solvents and, when



(A)



(B)



(C)

Fig. 1. Cells were treated with 0.5 mM H_2O_2 for indicated times in the presence or absence of Extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis*. After treatment, cell viability was estimated by MTT assay. (A) Cells were treated with 0.5 mM H_2O_2 for indicated times. (B) Cells were treated with 1.25, 2.5, 5, 10 ug/mℓ extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* for 24 hours. (C) Exposure to 0.5 mM H_2O_2 and different dose of extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* (1.25, 2.5, 5, 10 ug/mℓ) for 24 hours. Values are the means ± SD of three separate experiments performed in triplicate. *p <0.01 vs. control; $^{\#}$p < 0.01 vs. H_2O_2 alone.

followed by a solubilization post-treatment (Sun *et al*. 2004). But, at severity log *Ro* 4.98 and severity log *Ro* 5.15, the amount of phenolic and flavonoid content were decreased.

Similarly, it has been reported that the amount of phenolics in *Sasa palmate* leaves was gradually increased with the increase of temperature from 180 to 250℃, but at a temperature of 260℃, the amount of phenolic compounds was decreased (Akihiro *et al*. 2007).

3.2. MTT assay of the extracts

Cell viability was detected by MTT assay which relies on the mitochondrial metabolic capacity of viable cells. Oxidative stress is known to promote fibroblast cell death. Thompson *et al*. (1998) reported that the relatively low concentrations of H_2O_2 caused maximal DNA fragmentation and more apoptotic cell death. In contrast, the higher concentrations of H_2O_2 caused less DNA fragmentation, more necrotic cell death. Cells were incubated with H_2O_2 at the indicated concentration for 24 hours, and then cell viability was measured using the MTT assay. The results in Fig. 1A show that application of H_2O_2 induced a time-dependent viability loss in tenofibroblast cells. The viability of cells incubated with H_2O_2 at concentration of 0.5 mM for 24 hours was 43% of the control value. To study the cytotoxic effect of the extract from severity log *Ro* 4.68 we employed the extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* (Fig. 1B). We treated tenofibroblast cells with various concentrations of

extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* for 24 hours. The cell viability did not show significant changes 1.25 to 10 ug/mℓ of extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis*. To study the cytoprotective effect of extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* against H_2O_2-induced cell death, cells were treated with extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* (1.25, 2.5, 5, 10 ug/mℓ) in the presence of 0.5 mM H_2O_2. The viabilities were significantly increased to 91%, 101%, 103%, and 109%, respectively (Fig. 1C). Kwak *et al.* (2007) reported that the direct antioxidant NAC (N-acetylcysteine) exerted a significant cytoprotective effect on H_2O_2- mediated Murine neuroblastoma Neuro2A cells death, in a somewhat concentration-dependent manner. The cell viability was restored to roughly 80 % of the control at a concentration of 2.5 mM. The cytoprotective effect is higher than that the report for antioxidant NAC. These results are demonstrated that oak extract do not have a cytotoxic effect on tenofibroblasts of the rat achilles. Instead, oak extract appear to have, on these tenofibroblasts, a protective effect against oxidative stress.

3.3. Flow cytometry analysis of the oak extracts

Fig. 2 showed that apoptotic rate of tenofibroblasts cells was increased to 73.49 ± 1.57 from 3.73 ± 0.31 in control groups (0 ug/mℓ)

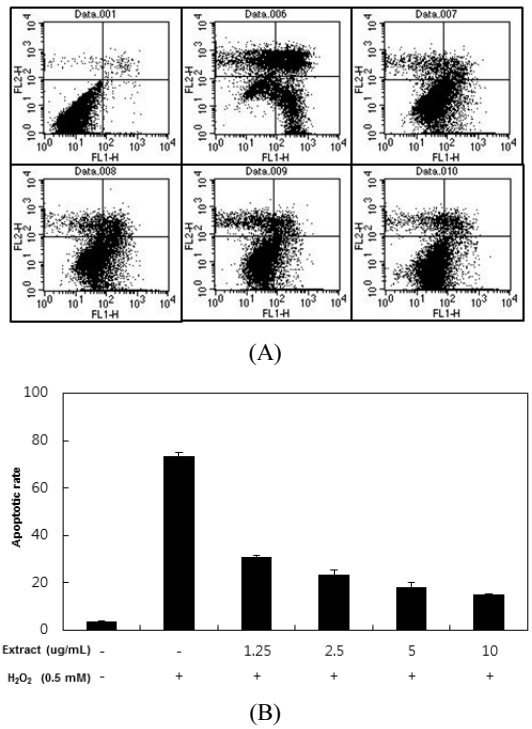


Fig. 2. Tenofibroblast cells were pretreated with 1.25, 2.5, 5, 10 ug/mℓ extract from steam exploded (severity log Ro 4.68) *Q. variabilis* for 1 hour before exposure to 0.5 mM H_2O_2 for 24 h. Then, cellular apoptosis was assayed by annexin V/PI double staining and analyzed by flow cytometry with fluorescence-activated cell sorting. The results were expressed as the original figures of flow cytometry (A) and apoptotic rate (B). Data were presented as mean ± S.D. of three independent experiments. Values are the means ± SD of three separate experiments performed in triplicate. *$p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. H_2O_2 alone.

after exposure of the cells to 0.5 mM H_2O_2 for 24 h ($p < 0.01$ *vs*. H_2O_2 groups). Preincubation of the cells with extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* at 1.25, 2.5, 5, 10 ug/mℓ reduced the apoptosis to 30.96 ± 0.64, 23.22 ± 2.11, 18.21 ± 2.01 and 14.88 ± 0.56, respectively. Chineses medicine

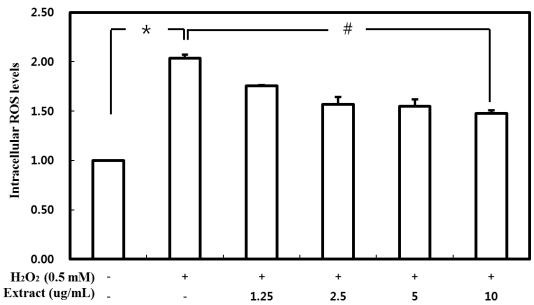


Fig. 3. Protective effect of extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* on H_2O_2-induced intracellular ROS. Intracellular ROS levels were determined by measuring DCF fluorescence, as described in Materials and methods. Values are the means ± SD of three separate experiments performed in triplicate. *$p < 0.05$ vs. control; #$p < 0.01$ vs. H_2O_2 alone.

Carthamus tinctorius L. (safflower) extract exerted a significant anti-apoptotic effect on H_2O_2- mediated neonatal cardiac cells death, in a somewhat concentration-dependent manner. Pretreatment with *Carthamus tinctorius* L. 100 mg/mℓ significantly reduced H_2O_2-induced apoptotic cells (17.11 ± 3.30 *vs* 32.10 ± 3.69) (Han *et al*. 2009). The anti-apoptotic effect of the oak extracts was higher than that the reported for *Carthamus tinctorius* L. extract. The oak extract-pretreated groups exhibited decreased H_2O_2-induced cytotoxicity and apoptotic cell death in a dose-dependent manner. The result indicates that oak extract inhibits H_2O_2-induced apoptosis of tenofibroblast cells.

3.4. Reactive oxygen species of the extracts

It is important to investigate the effect of extract on ROS production in the presence or absence of H_2O_2 treatment. The treatment of tenofibroblast cells with 0.5 mM H_2O_2 was found to increase intracellular ROS. That was significantly reduced by extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* /H_2O_2 (Fig. 3). The extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis*-pretreated group produced markedly less ROS than the H_2O_2-treated group. Park *et al*. (2010) examined effects of anthocyanins on H_2O_2-treated tenofibroblasts. The level of intracellular ROS production in the 100 ug/mℓ-anthocyanin-H_2O_2-treated group was significantly lower than the levels in the H_2O_2-treated group and in the control group (0 ug/mℓ). Oak extract ROS preventive effect was higher than that reported for anthocyanin. We examined the preventive effect of oak extract on H_2O_2-induced intercellular ROS production.

3.5. Western blot analysis of the extracts

Oxidative stress can stimulate MAPK signaling pathways that are closely associated with cell- survival and cell- death pathways. Generally, the activation of ERK cascades sustains cell viability, whereas the activation of JNK and p38 cascades promotes apoptosis. Experimental studies on the contrasting effects of these MAPKs have shown that the activation of each signaling pathway depends on its cell types and on the specific stimuli. To further analyze the effects of oak extract on the in-

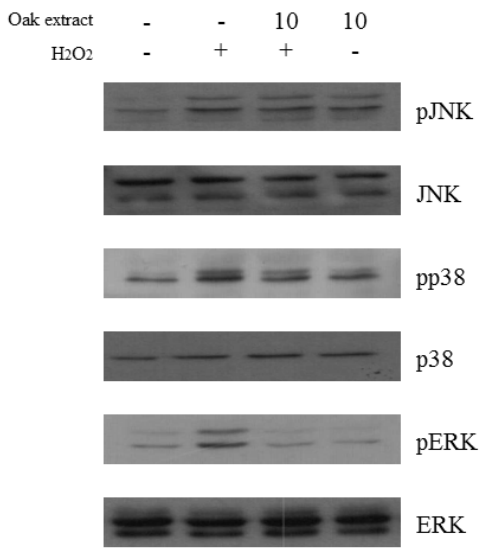


Fig. 4. Extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis* inhibited the H_2O_2 induced activations of ERK and p38 MAP kinase. Immunoblots of the lysates of treated rat achilles tenofibroblast cells were probed with antibodies for ERK, JNK, and p38 MAP kinase and with antibodies for their phosphorylated forms, respectively.

hibition of apoptosis, the effects of oak extract on MAPK (ERK, p38 and JNK) signaling pathways were studied by immunoblot analysis. Tenofibroblasts cells were exposed to H_2O_2 (0.5 mM) for 1 hour with or without oak extract and then the phosphorylation levels for ERK, p38 and JNK were examined. Oak extract (10 ug/mℓ) inhibited the H_2O_2-dependent phosphorylation of ERK and p38. However, oak extract did not change the phosphorylation levels of JNK (Fig. 4). In this study, we observed that the protective effects of oak extract against H_2O_2-induced apoptosis involve the blocking of the activation of ERK and p38. The cytoprotective effect of oak extract may, therefore, be the antiapoptotic result of the inhibition of the production of the ROS necessary to the activation of p38 and ERK. Dual activation of p38 and ERK might depend on the length of H_2O_2 exposure, the concentration of H_2O_2, and the type of cells. Experimental studies on the contrasting effects of these MAPKs have shown that the activation of each signaling pathway depends on its cell types and on the specific stimuli. Park *et al*. (2012) described that H_2O_2 caused significant activation of ERK and p38 protein, while *Juglans mandshurica* leaves extract at 200 ug/mℓ decreased levels of ERK, p38 protein.

3.6. Chemical composition of the extract

We characterized the chemical composition of ethanol extracts from steam exploded (severity log *Ro* 4.68) *Q. variabilis* using GC-MS. As shown in Table 3, the major chemical compounds found in extracts were 1,8-cineole (10.5%), vanillylmandelic acid (8.1%), crotonic acid (7.4%) and cyclopropanecarboxylic acid (6.8%).

Singh *et al*. (2009) reported that 1,8-cineole showed exhibited potent antioxidant activity, such as free radical (DPPH · , OH · , O2 · −) scavenging activity. This source of 1,8-cineole, also known as eucalyptol, exhibits anti-microbial activity (Hendry *et al*. 2009) and shows delivery-enhancing activity across the dermal barrier. Also, 1,8-cineole is used as an insecticide and insect repellent (Klocke *et al*. 1987; Sfara *et al*. 2009).

Table 3. Chemical composition of the extract from steam exploded (severity log *Ro* 4.68) *Q. variabilis*

No	Constituent	R.T[1)] (min)	Area (%)
1	Hydroxylamine	2.72	5.5
2	3-hydroxybromazepam	2.98	2.4
3	Methyl methacrylate	5.29	6.0
4	8-phthalimido-6-methoxy-2-[1-methyl-imidazol-2-yl]quinoline	8.05	5.3
5	Cyclopropanecarboxylic acid	9.07	6.8
6	Spheroidenone	10.81	3.1
7	Crotonic acid	12.95	7.4
8	7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride	18.28	4.7
9	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-	23.13	5.1
10	Norepinephrine	23.90	5.6
11	1,8-cineole	25.19	10.5
12	Vanillylmandelic acid	26.87	8.1
13	(1R)-(-)-Nopol benzyl ether	31.48	5.1
14	2',4'-Dihydroxy-3'-methylbutyrophenone	32.60	5.4
15	3-Carbobenzyloxy-4-ketoproline	36.80	5.8
	Unknown		13.2

[1)] R.T : Retention time

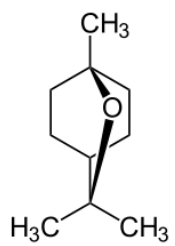


Fig. 5. Structures of 1,8-cineole.

Thus, it is believed that it is accordingly believed that the anti-apoptotic of the extracts is due mainly to the presence of 1,8-cineole (Fig. 5).

Further investigations examining the synergistic effect of 1,8-cineole and which one is minor components on the anti-apoptotic activity of the extracts are desirable.

4. CONCLUSION

These results have shown that ethanol extract from steam exploded *Q. variabilis* (severity log *Ro*) contain phenolic compounds. The main compounds in the ethanol extracts were 1,8-cineole (10.5%), vanillylmandelic acid (8.1%), crotonic acid (7.4%) and cyclopropanecarboxylic acid.

Recent research has shown that apoptosis is involved in tendon degeneration. This motivated researchers to focus on a possible role of apoptosis in the mechanism of achilles degeneration. H_2O_2 treatment alone significantly decreased cell viability to about 43%. However, when cells were pretreated with 1.25-10 ug/mℓ *Q. variabilis* extract for 1 hour, the cell viability

was restored to roughly 109% at a concentration of 10 ug/mℓ. According to the flow cytometry analysis, the *Q. variabilis* extract-pretreated groups exhibited decreased H_2O_2-induced cytotoxicity and apoptotic cell death in a dose-dependent manner. Also, the *Q. variabilis* extract-pretreated group produced markedly less ROS than the H_2O_2-treated group. The cytoprotective effects of *Q. variabilis* extract on the expression levels of ERK and p38 in tenofibroblasts cells in the response to H_2O_2 treatment.

In conclusion, the present study demonstrates that this *Q. variabilis* extract exerts a significant cytoprotective effect against H_2O_2-induced apoptosis in the tenofibroblasts cell. In view of these data, it can be concluded that 1,8-cineole (10.5%) has cytoprotective effects in H_2O_2-induced cell toxicity.

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