**Flavonoids from *Taraxacum coreanum* protect from radical-induced oxidative damage**

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The active components and protective activity of *Taraxacum coreanum* from oxidative stress under *in vitro* and cellular system using LLC-PK₁ renal epithelial cells were investigated. *T. coreanum* was extracted with methanol (MeOH) and then fractionated into four different layers, *n*-hexane, trichloromethane (CHCl₃), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). *In vitro*, the scavenging activities of the extract and fractions from *T. coreanum* and its active components, luteolin and luteoloside, on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and hydroxyl radical (·OH) were measured. In LLC-PK₁ cellular model, the protective activity of the EtOAc fraction, and the active components from oxidative stress induced by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), a generator of peroxyl radicals, were studied. Among the fractions, the EtOAc fraction and its active components, luteolin and luteoloside, exerted the strongest protective effect from DPPH and ·OH. Furthermore, the LLC-PK₁ cells showed a decrease in cell viability and an increase in lipid peroxidation by the treatment of AAPH. However, the EtOAc fraction and its active components led to the significant increases in the cell viability and inhibition in lipid peroxidation. The present results indicated that *T. coreanum* and its active components, luteolin and luteoloside, are promising antioxidants with the protective effect from oxidative stress induced by overproduction of free radical.

**Key words**: Oxidative stress, free radical, LLC-PK₁ cell, *Taraxacum coreanum*, luteolin, luteoloside.

**INTRODUCTION**

Reactive oxygen species (ROS)-induced oxidative stress leads to the modification of DNA, cellular proteins and membrane lipids; therefore, it plays a crucial role in a wide range of diseases and age-related degenerative conditions including cardiovascular diseases, inflammatory conditions and neurodegenerative diseases such as Alzheimer’s disease and cancer (Bokov et al., 2004; Halliwell, 1997). This relationship has led to considerable interest in searching for antioxidants to scavenge free radicals and elevate defense activity in biological systems. Although several synthetic antioxidants have been suggested for the prevention and treatment of diseases, some side effects and toxicities have become an issue. Natural antioxidants, because of relatively low toxicity and side effects compared with synthetic antioxidants, have attracted much attention as preventive and therapeutic agents for attenuating oxidative damage.

Plants of the genus *Taraxacum* also known as dandelions, are in the family of the Asteraceae and have long been used as medicinal herbs. The name is derived from the Greek words “taraxis”, for inflammation, and “akeomai”, for curative. *Taraxacum coreanum*, a native plant of Korea, grows chiefly in the Korea and China. *T. coreanum* has been used as diuretic drugs and anti-inflammatory medicines (Ahn, 1998; Koo et al., 2004). The functions of *T. coreanum* are related to its phytochemical compounds including phenols and flavonoids,
The electron ionization-mass spectrometry (EI-MS) was measured using a JEOL JMS-600W (Japan) mass spectrometer, and the fast atom bombardment-mass spectrometry (FAB-MS) was measured with a JEOL JMS-AX505WA (Japan) mass spectrometer. 1H- and 13C-NMR spectra were recorded with a Bruker AVANCE 300 NMR (Germany) spectrometer using tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in parts per million (δ), and coupling constants (J) were expressed in hertz.

Evaporation was conducted using an EYELA rotary evaporator (Japan) under reflux in vacuo. Thin layer chromatography (TLC) was conducted with Kiesel gel 60 F254 (Art. 5715, Merck Co., Germany) plates (silica gel, 0.25 mm layer thickness). Recycling preparative high performance liquid chromatography (HPLC) was conducted by a JAI LC-9014 system and determination was performed by an L-6050 system pump with a UV-3702 system UV/VIS detector.

**Extraction, fractionation and identification**

Dried and finely powdered aerial parts of *T. coreanum* (1970 g) were extracted with MeOH for 3 h (4 L × 8) under reflux at 65–75 °C, and the solvent was evaporated in vacuo to isolate the methanol (MeOH) extract (693 g). This extract was suspended in distilled water and partitioned with n-hexane, trichloromethane (CHCl3), ethyl acetate (EtOAc), and n-butanol (n-BuOH), successively. Each fraction such as n-hexane (99 g), CHCl3 (12 g), EtOAc (23 g), and n-BuOH (25 g) fractions was obtained. A portion of the EtOAc fraction (7.0 g) was performed to a silica gel column chromatography (6 × 80 cm, No. 7734), with a gradient of n-hexane–EtOAc (100% n-hexane up to 100% EtOAc) and EtOAc–MeOH (EtOAc–MeOH mixture of increasing polarity) to yield 16 subfractions. Subfractions 7 and 12 eluted with 30 and 100% EtOAC in n-hexane, respectively, yielded luteolin and luteoloside, respectively (Figure 1) (Lee et al., 2011).

**Reagents**

1,1-Diphenyl-2-picrylhydrazyl (DPPH), malondialdehyde (MDA), and 2-deoxyribose were purchased from Sigma (Sigma-Aldrich, Korea). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's modified eagle's medium/nutrient mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) was purchased from Hyclone (Grand Island, NY, U.S.A.) and GIBCO (Cleveland, OH, U.S.A.), respectively.

**General instruments**

The electron ionization-mass spectrometry (EI-MS) was measured with a JEOL JMS-600W (Japan) mass spectrometer, and the fast atom bombardment-mass spectrometry (FAB-MS) was measured with a JEOL JMS-A505WA (Japan) mass spectrometer. 1H and 13C-NMR spectra were recorded with a Bruker AVANCE 300 NMR (Germany) spectrometer using tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in parts per million (δ), and coupling constants (J) were expressed in hertz.
well plates at 10

After confluence had been reached, the cells were seeded into 96-

Cells were sub-cultured weekly with 0.05% trypsin–EDTA in PBS.

FeSO$_4$ microplate reader (model SPECTRA max 340PC, Molecular

room temperature, the DPPH radicals were determined using a

Hatano et al. (1989). After being mixed gently and left for 30 min at

ethanolic solution of DPPH (60 mM) according to the method of

H$_2$O$_2$)

In a microwell plate, 100 µL of sample solution. The reaction was initiated by the addition of

H$_2$O$_2$. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% trichloroacetic acid (TCA) and 0.75 ml of 1.0% of 2-tribarbituric acid in 50 ml of NaOH. The solution was boiled for 10 min, and then cooled on ice. Finally, the absorbance of

the solution was measured at 520 nm. Hydroxyl radical scavenging activity was evaluated as the inhibition rate of 2-deoxyribose oxidation by ·OH (Gutteridge, 1987).

Cell viability was assessed using the MTT colorimetric assay. MTT

assay

Cell viability was assessed using the MTT colorimetric assay. MTT solution (1 mg/ml) was added to each 96-well culture plate and incubated for 4 h at 37°C, and then the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 ml of dimethyl sulfoxide (DMSO) and the absorbance of each well was read at 540 nm using a microplate reader.

Measurement of thiobarbituric acid reactive substances (TBARS)

The level of lipid peroxidant released from the cultured cells was estimated as TBARS according to the methods of Yagi (1976) and Yokode et al. (1988) with slight modifications. One aliquot of medium was mixed with 1.5 ml of 0.67% TBA solution and 1.5 ml of 20% TCA, and boiled at 95 - 100°C for 45 min. The mixture was cooled with water and shaken vigorously with 3.0 ml of n-butanol. After the mixture was centrifuged at 4000 X g for 10 min, the n-butanol layer was removed, and the absorbance was measured at 520 nm on a fluorescence spectrophotometer (Model FR-550, Shimadzu, Kyoto, Japan).

Statistical analysis

All statistical analyses were assessed by SAS software (SAS Institute, Cary, NC, USA). P < 0.05 was determined as statistically significant. Measurement data (n = 6) were expressed as mean ± standard deviation.

RESULTS

DPH scavenging activity

The MeOH extracts and 4 fractions from T. coreanum showed strong DPH radical scavenging activity (Table 1). Among all fractions, the EtOAc fraction showed the strongest DPHH radical scavenging activity. Based on this result of DPHH radical, we investigated the radical scavenging activities of EtOAc fraction from T. coreanum. The treatment of the EtOAc fraction from T. coreanum increased DPHH radical scavenging activity in a dose-dependent manner (Table 2). At the 100 mg/ml concentration, 90% inhibition of DPHH radical was observed.

-OH scavenging activity

The EtOAc fraction also had the strong protective effect against ·OH in a dose-dependent manner (Table 3). At the concentrations of 0.5, 2.5, 50 and 100 µg/ml, the EtOAc fraction showed 87.9, 89.9, 90.2 and 90.4% of ·OH scavenging activity, respectively.

Protective activity against peroxyl radical-induced oxidative stress

The viability of LLC-PK$_1$ renal epithelial cells treated with AAPH was reduced to 35.4% by the treatment with 1 mM AAPH for 24 h (Figure 2). However, the treatment of the EtOAc fraction exerted protective activity against AAPH-induced cellular damage. When the EtOAc fraction was treated at the dose of 100 µg/ml, the cell viability was elevated to 80%.

Table 1. IC$_{50}$ values of the MeOH extract and 4 fractions from T. coreanum against DPPH radical.

<table>
<thead>
<tr>
<th>Group</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>48.74 ± 0.98*</td>
</tr>
<tr>
<td>n-hexane</td>
<td>3.87 ± 0.24&lt;</td>
</tr>
<tr>
<td>CHCl$_3$</td>
<td>11.09 ± 0.16&lt;</td>
</tr>
<tr>
<td>EtOAc</td>
<td>0.00003 ± 0.00&lt;</td>
</tr>
<tr>
<td>n-BuOH</td>
<td>0.09 ± 0.00&lt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *Means with the different letters are significantly different (P<0.05) by Duncan's multiple range test.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Hydroxyl radical (·OH) scavenging activity

In a microwell plate, 100 µL of fractions and flavonoids from T. coreanum (control: 100 µL of distilled water) were added to an ethanolic solution of DPPH (60 mM) according to the method of Hatano et al. (1989). After being mixed gently and left for 30 min at room temperature, the DPPH radicals were determined using a microplate reader (model SPECTRA max 340PC, Molecular Devices, Sunnyvale, CA, U.S.A.).
Table 2. DPPH and ·OH scavenging activity of the EtOAc fraction from *T. coreanum*.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Scavenging activity (%)</th>
<th>DPPH</th>
<th>·OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>82.68 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.87 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>85.83 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.89 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>89.37 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.20 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>89.76 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.44 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD. Means with the different letters are significantly different (*P*<0.05) by Duncan's multiple range test.

Table 3. DPPH radical scavenging activity of flavonoids from *T. coreanum*.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Scavenging activity (%)</th>
<th>Luteolin</th>
<th>Luteoloside</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>68.90 ± 0.79&lt;sup&gt;d&lt;/sup&gt;</td>
<td>74.80 ± 0.79&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>77.56 ± 0.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79.13 ± 0.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>81.89 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.80 ± 0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>90.55 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.28 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>0.05 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD. Means with the different letters are significantly different (*P*<0.05) by Duncan's multiple range test.

**Figure 2.** Effect of the EtOAc fraction from *T. coreanum* on viability of LLC-PK<sub>1</sub> cells treated with AAPH. Values are mean ± SD. Means with the different letters are significantly different (*P*<0.05) by Duncan's multiple range test.

**Inhibition of lipid peroxidation against peroxyl radical-induced oxidative stress**

AAPH led to an increase of lipid peroxidation in LLC-PK<sub>1</sub> renal tubular epithelial cells, whereas the EtOAc fraction significantly decreased the formation of TBARS in a concentration-dependent manner (Figure 3). Although 0.734 nmol/mg protein of TBARS was produced in cells treated only with AAPH, the treatment with the EtOAc fraction at the 100 µg/ml decreased lipid peroxidation to
Figure 3. Effect of the EtOAc fraction from T. coreanum on lipid peroxidation of LLC-PK₁ cells treated with AAPH. Values are mean ± SD. **Means with the different letters are significantly different (P<0.05) by Duncan's multiple range test.

0.402 nmol/mg protein (45.2% decrease).

Antioxidative activity of luteolin and luteoloside

The IC₅₀ values of the DPPH radical scavenging effects of flavonoids, luteolin and luteoloside, were 0.05 and 0.03 µg/ml, respectively (Table 3). In addition, as revealed in Figure 4, AAPH treatment reduced LLC-PK₁ cell viability to 32.2%. Treatment with both luteolin and luteoloside was able to recover the cellular damage induced by AAPH in a dose-dependent manner, and at the concentration of 10 µg/ml, the cell viability was elevated to 90.8 and 95.1%, respectively. In addition, luteolin and luteoloside also showed protective effects against lipid peroxidation induced by peroxyl radicals (Figure 5). Peroxyl radicals increased the formation of MDA from 0.50 nmol MDA/mg protein to 0.95 nmol MDA/mg protein. The treatment of luteolin and luteoloside significantly inhibited lipid peroxidation, particularly at 10 µg/ml, and MDA level was decreased to 0.502 (46% decrease) and 0.499 nmol MDA/mg protein (45% decrease), respectively.

DISCUSSION

Free radical-mediated oxidative stress results in a variety of pathological conditions (Bokov et al., 2004; Halliwell, 1997). Therefore, antioxidants that prevent damage caused by free radicals are considered to be worthy of study. T. coreanum is a well-known traditional herbal medicine with a long history. Until recently, only limited scientific information has been available to justify its reputed uses (Gurib-Fakim, 2006). Therefore, the present investigation was focused on the protective activity of T. coreanum and its active components, luteolin and luteoloside, from free radical-induced oxidative stress in both in vitro and cellular system.

We tested the radical scavenging activity of the MeOH extract and 4 fractions of T. coreanum against DPPH and ·OH scavenging activities. DPPH is a stable free radical and has been widely used to test the ability of compounds or plant extracts to act as free radical scavengers or hydrogen donors (Lee et al., 2011; Zhu et al., 2002). Antioxidants directly react with the DPPH radical and restore it by transfer of electrons or hydrogen. Therefore, we used this system for assessing the radical scavenging activity of the MeOH extract and 4 fractions of T. coreanum. In the DPPH scavenging activity tests of the MeOH extract and 4 fractions of T. coreanum, the EtOAc fraction showed the strongest DPPH radical scavenging activity. Based on the DPPH result, we investigated the radical scavenging activities of the EtOAc fraction from T. coreanum. The treatment with the EtOAc fraction increased DPPH radical scavenging activity in a dose-dependent manner. These results suggest that the EtOAc fraction from T. coreanum includes the promising agents for scavenging of free radicals.

·OH induces various injuries to the surrounding organs and plays a vital role in some clinical disorders. Therefore, removal of ·OH is the most effective defense of living body against diseases (Lin et al., 1995). In particular, among various different radicals, the ·OH is an extremely
reactive and short-lived species that can attack biological molecules such as DNA, proteins, and lipids. The reactivity of ·OH has been related to several human diseases such as neurodegenerative disease and
diabetes. Therefore, its scavenging activity has received much attention (Halliwell and Gutteridge, 1984; Halliwell et al., 1992; Zhang et al., 1996). The ETOAc fraction showed the strongest protective effect against ·OH in a dose-dependent manner. From these results, we confirmed that the ETOAc fraction from T. coreanum may contain an effective ·OH scavenger capable of protecting against radical-induced oxidative damage. The present results suggest that the ETOAc fraction may play a protective role against free radical-induced oxidative stress.

Meanwhile, the reactions of free radicals in biological systems are complicated. To study these reactions, a well-designed in vitro model system is required. Thermal decompositions of free radical initiators, including peroxides, hyponitrites, and azo compounds induce oxidative stress. To generate free radicals at a known, constant and well-defined rate, thermal decomposition of free radical initiators is preferred. AAPH, one of the hydrophobic azo compounds, generates free radicals at a constant and measurable rate by its thermal decomposition without biotransformation (Terao and Niki, 1986). The free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxyl radicals. The lipid peroxyl radicals in turn attack other lipid molecules to form lipid hydroperoxide and new lipid radicals. This reaction takes place repeatedly with resultant attacks upon various biological molecules, and induces physiochemical alterations and cellular damage (Miki et al., 1987). Finally, AAPH causes a diverse array of pathological changes. Therefore, AAPH-intoxication experiments are useful for evaluating biological activities of antioxidants. To investigate the antioxidative activity of the ETOAc fraction from T. coreanum in a cellular system using LLC-PK₁ renal tubular epithelial cells that are susceptible to oxidative stress, we employed such an AAPH model system.

Several reports have documented that AAPH decreased the viability of hepatic and neuron cells (Matsura et al., 1992; Rapin et al., 1998). The treatment with AAPH induces apoptosis in the cells, causing loss of viability. The present study also shows that AAPH leads to a decline in viability of LLC-PK₁ renal epithelial cells. However, our results demonstrated that the ETOAc fraction exerted a protective effect against oxidative damage by AAPH to LLC-PK₁ cells, resulting in increased cell viability in a dose dependent manner. It is well accepted that lipid peroxidation in biological systems is toxicological phenomenon, resulting in pathological consequences (Hochstein and Jain, 1981). Therefore, measurement of lipid peroxidation end products such as TBARS provides a good index of cell destruction. Our results revealed that AAPH treatment increased the formation of TBARS in LLC-PK₁ renal epithelial cells, indicating cellular damage by AAPH. However, the treatment with the ETOAc fraction also showed decrease in AAPH-induced lipid peroxidation. These results indicate that T. coreanum exerted protective activity from AAPH-induced cell injury and lipid peroxidation by scavenging peroxyl radicals generated from AAPH, suggesting the roles as promising antioxidants.

The present study clearly demonstrates that luteolin and luteoloside primarily are responsible for the radical scavenging effect and protective activity from oxidative damage. Several studies have demonstrated that Taraxacum officinal extracts possess antibiotic activities, anti-oxidative properties, anti-inflammatory and anti-tumor substances (Kimura et al., 1985; Wagner, 1989). Moreover, T. officinal contains terpenoid and sterol compounds such as taraxacin and taraxacerin, which are distributed equally in the roots, leaves and flowers. Other terpene/sterol compounds include β-amyrin, taraxasterol, taraxerol, sitosterin, stigmasterin, and phytosterin. However, the active compounds from T. coreanum with antioxidative effect have not been identified yet. We previously isolated and identified the active components from T. coreanum, luteolin and luteolin-7-glucoside (Lee et al., 2011). Our present study supported the protective role of luteolin and luteoloside from oxidative stress induced by free radicals.

In conclusion, T. coreanum exhibited radical scavenging activity in vitro and antioxidative activity against oxidative stress in a cellular model. In addition, luteolin and luteoloside isolated from T. coreanum have been shown to have antioxidative activity. Although further study on the protective mechanisms of these compounds is necessary, the present study supports the promising role of T. coreanum as a source of antioxidative compounds against free radical-induced oxidative stress.

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REFERENCES

Hatano T, Edamatsu R, Hiramatsu M, Mori A, Fujita Y, Yasuhara T,


