The ethanol extract from the fruit of *Terminalia chebula* (Combretaceae) exhibited significant inhibitory activity on oxidative stress and the age-dependent shortening of the telomeric DNA length. In the peroxidation model using t-BuOOH, the *T. chebula* extract showed a notable cytoprotective effect on the HEK-N/F cells with 60.5 ± 3.8% at a concentration of 50 µg/ml. In addition, the *T. chebula* extract exhibited a significant cytoprotective effect against UVB-induced oxidative damage. The life-span of the HEK-N/F cells was elongated by 40%, as a result of the continuous administration of 3 µg/ml of the *T. chebula* extract compared to that of the control. These observations were attributed to the inhibitory effect of the *T. chebula* extract on the age-dependent shortening of the telomere, length as shown by the Southern blots of the terminal restriction fragments (TRFs) of DNA extracted from subculture passages. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: *Terminalia chebula*; Combretaceae; oxidative stress; telomere length; Southern blots.

INTRODUCTION

The fruit of *Terminalia chebula* Retzius (Combretaceae), which is commonly known as black myrobalan, is a popular medicinal plant that has been used to treat diarrhea, dysentery, leukorrhea, hemorrhage, prolapse of the rectum and chronic cough (Namba, 1993). In addition, antimicrobial (Malekzadeh et al., 2001), antiviral (Yukawa et al., 1996; Ahn et al., 2002), anticancer (Lee et al., 1995; Saleem et al., 2002), anti-anaphylaxis (Shin et al., 2001) and anti-diabetic (Sabu and Kuttan, 2002) activities have been reported.

It is well known that reactive oxygen species (ROS) generated in the metabolic pathway damage biomolecules such as lipids, proteins, sugars and DNA, which can not only lead to various diseases (Halliwell, 1987; Vishwanath, 1995), but also oxidative damage resulting aging (Barja, 2002; Sohal et al., 2002). Indeed, oxidized biomolecules such as the 8-oxo-2′-deoxyguanosine residues in DNA (Ozawa, 1995; Lezza et al., 1999), carbonyls and dityrosines in proteins (Berlett and Stadtman, 1997; Leeuwenburgh et al., 1997) and hydroperoxides in lipids (Tahara et al., 2001) have been reported to accumulate in the tissues of aged animals. This oxidative stress theory of aging is supported by many studies, and it is now accepted one of the most important theories of aging (Barja, 2002; Sohal et al., 2002). Accordingly, antioxidants, which can prevent oxidative damage from ROS, are expected to inhibit the aging process. Recently, another theory, the telomere hypo-

* Correspondence to: Prof. K. Bae, College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea.
E-mail: baekh@cnu.ac.kr

Copyright © 2004 John Wiley & Sons, Ltd.

MATERIALS AND METHODS

Materials and preparation of extracts

The dried fruit from *T. chebula* was supplied from a pharmaceutical company in Korea, HanKookSinYak Co., Ltd. (Nonsan, Korea), in April 2001 and was identified by Professor KiHwan Bae, College of Pharmacy, Chungnam National University. A voucher specimen...
Cells grown at \(1 \times 10^4 \text{cells/100} \mu \text{l}\) of DMSO or a medium for bioassay.

**Oxidative stress induced cell damage**

**Cell Culture.** The human epidermal keratinocytes-Neonatal/Foreskin (HEK-N/F) were purchased from Modern Tissue Technologies, Inc. (MC1312, Seoul, Korea). The HEK-N/F cells were cultured in a type IV collagen coated plate with KGM\(^{\text{®}}\) Bulletkit medium (CC-3111, Clonetics San Diego) in humidified atmosphere of 5% \(\text{CO}_2/95\)% air at 37 °C, and cultured to 90% confluence.

\(t\)-BuOOH induced oxidative stress. The HEK-N/F cells (1 \times 10^4 \text{cells/100} \mu \text{l}) were seeded on a 96-well microplate and precultured for 24 h. The cells were then treated with 1 \mu l of the \text{T. chebula} extract and 10 \mu l of \text{t-BuOOH} (1.5 mM) dissolved in Hank’s balanced salt solution (HBSS, Gibco. BRL, New York) for 3 h in order to induce cellular peroxidation. The cell viability was measured using the methylthiazolotetrazolium (MTT) method. Briefly, the MTT (5 mg/ml) dissolved in phosphate buffered saline (PBS) was added to comprise less than 10% of the total volume. After 4 h incubation, the remaining medium was aspirated and 100 \mu l of DMSO was added to dissolve the formazan formed from the MTT. The absorbance was read at a wavelength of 50 °C with the oligodeoxynucleotide probe 5′-[\(^{32}\)P]-labeled (TTAGGG)\(_4\) (TaKaRa). The membranes were washed with in 4 \times \text{SSC/0.1% sodium dodecyl sulfate (SDS)} at 35 °C and underwent autoradiography on X-ray film (Scientific imaging film, Kodak), followed by densitometry using a laser densitometer UltraScan XL (Pharmacia).

**UV irradiation induced oxidative stress.** Cells grown at the logarithmic phase were seeded at density of 0.5–4 \times 10^4 \text{cells/cm}^2 in a 24-well microplate in order to allow the cells to proliferate to the subconfluent state upon UV irradiation. After 18 h, the \text{T. chebula} extract was administered for 24 h. After rinsing with PBS, the cells were irradiated with a Spectronics UV transilluminator EBF-260 (the maximum wavelength, 312 nm; a half-peak intensity range, 297–328 nm). The UV-irradiated cells were cultured for 1–7 h in fresh medium, and treated with 5 \mu M ethidium homodimer (Et\(_2\), Millipore). After 30 min, the fluorescent intensity of the 24-well microplate was determined using a fluorescence plate reader CytoFluor 2350 (Millipore, Danvers, MA) at 485 nm for excitation and 645 nm for emission (Kanatate et al., 1995).

**RESULTS AND DISCUSSION**

**Cytoprotective effect of the \text{T. chebula} extract on the \text{t-BuOOH} induced oxidative stress**

\text{t-BuOOH} is a hydroperoxidant that can be metabolized to free radical intermediates, which can subsequently initiate lipid peroxidation resulting in cell damage. The cytoprotective effect of the \text{T. chebula} extract induced by \text{t-BuOOH} is shown in Table 1. The cell viability of the HEK-N/F cells was significantly decreased to 11.2 ± 1.2% when they were treated with 1.5 mM \text{t-BuOOH}

<table>
<thead>
<tr>
<th>Sample treatment (\mu g/ml)</th>
<th>Cell viability (%)</th>
<th>TBARS (pmole MDA/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.2 ± 1.2</td>
<td>359.2 ± 65.8**</td>
</tr>
<tr>
<td>\text{t-BuOOH}</td>
<td>25.0</td>
<td>9024.5 ± 890.1</td>
</tr>
<tr>
<td>+ \text{T. chebula extract}</td>
<td>50.0</td>
<td>1010.7 ± 117.5**</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1867.5 ± 201.4**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of triplicate experiments.

* \(P < 0.05\), ** \(P < 0.001\), were significantly different from the group treated with \text{t-BuOOH}.  

---

Table 1. Effect of the \text{T. chebula} extract on the \text{t-BuOOH} induced oxidative damage in the HEK-N/F cells

---

Copyright © 2004 John Wiley & Sons, Ltd.

for 3 h, whereas the cell viability was increased to 60.5 ± 3.8% when they were treated with the *T. chebula* extract at a concentration of 50 µg/ml. There was also a dose-dependent relationship (Table 1), suggesting that the *T. chebula* extract protected the cells from oxidant-induced cell damage in terms of its ability to scavenge the t-BuOOH derived ROS. The cytoprotective effect of the *T. chebula* extract against the t-BuOOH induced oxidative stress was also confirmed by determining the level of lipid peroxidation (Table 1). The TBA reactive substance (TBARS) of the cells treated with t-BuOOH (1.5 mM) for 3 h was increased 25-fold compared to the untreated control. As shown in Table 1, the TBARS of the cells treated with the *T. chebula* extract was also significantly decreased, which means that the *T. chebula* extract could protect the cells from oxidative stress.

**Cytoprotective effect of the *T. chebula* extract on the UVB irradiation**

It is well known that UV irradiation produces DNA strand breaks, DNA-protein crosslinks and base modifications mediated by ROS, which can result in skin cancer, inflammation and photoaging (Black *et al.*, 1997). When the HEK-N/F cells were irradiated with the UV B of 35 mJ/cm², the DNA strands of the nucleus was cleaved, which was quantified by the fluorescence intensity of Et₂, which is a dead cell specific DNA strand intercalating fluorescent dye (Fig. 1A). As shown in Fig. 1B, the fluorescence intensity of the *T. chebula* extract (50 µg/ml)-added cells was 50% lower than that of the untreated, irradiated control. That is, the UV B-induced cleavage of the nuclear DNA strands was markedly prevented by the *T. chebula* extract.

**Elongating effect on cellular life-span**

Most mammalian cells cultivated in *vitro* undergo a limited number of cell divisions and then arrest in a stage known as replicative senescence. In a report (Furumoto *et al.*, 1998), it was found that cultivating the cells under enhanced oxidative stress accelerates the replicative senescence, and antioxidants could elongate the life span. To investigate the effect of the *T. chebula* extract on the cellular doubling potential, the HEK-N/F cells were serially subcultured until the spontaneous stoppage of cell division in the presence or absence of the *T. chebula* extract. The untreated cells rapidly grew to a PDL of 14.9, whereas 3 µg/ml of the *T. chebula* extract enhanced the maximum PDL to 20.9. The cellular life was extended 1.40-fold in comparison to the control group (Fig. 2).

**Inhibitory effect of age-dependent telomere shortening**

Based on the telomere theory of aging, the elongating effect of the cellular life span was evaluated by measuring the telomere length in each culture passage. The DNA extracted from the HEK-N/F cells of each culture passage was restricted by *Hinf* I to produce the TRFs, which were analyzed by Southern blot analysis. As shown in Fig. 3, the telomeres in the untreated control cells gradually shortened from 12.0 kb (PDL 2.8) to 7.9 kb (PDL 14.9). In comparison to this, when the *T. chebula* extract was continuously administered to the cells, the telomere length shortening was inhibited. At PDLs of 14.9 when the telomere length in the control cells reached its minimum, the *T. chebula* extract added cells retained telomeres that were approximately 1.7 kb longer than those of the untreated cells. It was observed that in the case of the cells administrated to the *T. chebula* extract, the telomere length had shortened by an average of 192 bp/PDL, which was slower than the 342 bp/PDL observed in the control group (Fig. 4). Cell division can be continuously promoted as far as the telomeric DNA is kept at more than a critical value (in this report, it is assumed to be approximately 8.0 kb). It was found that the *T. chebula* extract decreased the shortening rate of the telomeric DNA, thereby delaying the rate to reach the critical value. It could therefore it extend the period for the termination of cell division.

---

*Figure 1. Cytoprotective effect of the *T. chebula* extract on the UVB-induced oxidative stress. The cells were treated with 50 µg/ml of the extract or not for 24 h, rinsed with PBS, and irradiated with UVB. After cultivation for 1–7 h with fresh medium, the cells were stained with 5 µM Et₂. Debris and membrane-disrupted cells were stained with Et₂ as appearing bright in the fluorescent microphotography (A). Relative fluorescence intensity was expressed as percentage of Et₂ fluorescence intensity of tested cells compared to the cells completely destroyed by ethanol (B).*
Figure 2. Elongating effect of the *T. chebula* extract on the lifespan in the HEK-N/F cells. Cellular life-spans were elongated from PDL 14.9 for control to PDL 20.9 for the *T. chebula* extract-added cells.

Figure 3. Dependence of the telomere length on the PDLs of the HEK-N/F cells serially subcultivated in the presence or absence of the *T. chebula* extract. The telomere length was determined by Southern blots using a [32P]-labeled (TTAGGG)_4 oligonucleotide probe for the TRFs of the genomic DNA extracted from each passage of the cells administered with or without 3 µg/ml of the *T. chebula* extract.

Figure 4. Dependence of the telomeric DNA length on the cellular ages (PDL) of the HEK-N/F cells administered with or without the *T. chebula* extract. Mean TRF length is estimated as a center of mass and expressed in kb based on the following equation: Σ(MWi × ODi)/Σ(ODi), where ODi is densitometric output and MWi is the length of the DNA at position i.

**Anti-aging effect of *T. chebula* based on the two aging theories**

The two aging theories, oxidative stress theory and telomere theory, have been established well by many related evidences. Recently, it was recognized that oxidative damage is repaired less well in the telomeric DNA than elsewhere in the chromosome, and oxidative stress accelerates telomere loss (Furumoto *et al.*, 1998; Lorenz *et al.*, 2001; Saretzki and von Zglinicki, 2002; von Zglinicki, 2002). Measuring both antioxidant activity on oxidative stress and telomere shortening rate, we found that the *T. chebula* extract could slowdown the telomere shortening rate by inhibition of oxidative stress. Therefore, it is suggested that the *T. chebula* fruit has an effect on the prevention of aging, so further studies of the active principle might be expected.

**Acknowledgements**

This research was supported by a grant of the Korea Health 21 R&D Project Ministry of Health and Welfare, Republic of Korea (01-PJ2-PG6-01NA01-0002) and research funds from HanKookSinYak Co. Ltd in Korea.

**REFERENCES**


