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Cytoprotective Effect on Oxidative Stress and Inhibitory Effect on Cellular Aging of *Terminalia chebula* Fruit

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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.

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 hypothesis of aging, has also been accepted together with the oxidative stress theory. Telomeres located at the end of linear chromosome shorten with each cell division, and when the telomeric DNA reaches a critical length, this DNA damage can ultimately lead to cell cycle arrest in senescent cells (Harley *et al*., 1992; Vaziri and Benchimol, 1996; Hornsby, 2002). Shortening of the telomeric DNA has been observed in human cells during the aging processes (Tsuji *et al*., 2002). A life span extension has been observed after introducing telomerase, which can maintain the telomere length, into normal human cells (Bodnar *et al*., 1998). The telomere hypothesis has been supported by many reports.

In our previous study, the fruit from *T. chebula* exhibited potent antioxidant activity on DPPH radical and lipid peroxidation (Na *et al*., 2001). Based on the two aging theories, this study investigated the cytoprotective activity of the *T. chebula* extract on the oxidative stress induced by tertiary butyl hydroperoxide (*t*-BuOOH) and ultraviolet-B (UVB) irradiation. Furthermore, the inhibitory effect of the medicinal plant on the cellular aging of human epidermal keratinocytes was examined by measuring the telomere length.

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

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(HK 001) was deposited in the Jakwang Research Institute of the Hansaeng Cosmetics Co., Ltd. Twenty grams of the dried fruit from *T. chebula* was extracted using 100 ml ethanol at room temperature for 2 weeks. The ethanol extract was concentrated and dissolved in 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® Bulletkit medium (CC-3111, Clonetics San Diego) in humidified atmosphere of 5% CO_2/95% air at 37 °C, and cultured to 90% confluence.

***t*-BuOOH induced oxidative stress.** The HEK-N/F cells (1×10^4 cells/100 µl) were seeded on a 96-well microplate and precultured for 24 h. The cells were then treated with 1 µl of the *T. chebula* extract and 10 µl of *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 methylthiazoletetrazolium (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 µl of DMSO was added to dissolve the formazan formed from the MTT. The absorbance was read at a wavelength of 570 nm. The inhibitory activity of lipid peroxidation was also determined using the thiobarbituric acid (TBA) method, as previously described (Park *et al*., 2000).

UV irradiation induced oxidative stress. Cells grown at the logarithmic phase were seeded at density of $0.5–4 \times 10^4$ cells/2 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 *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 µM 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).

Inhibitory activity of telomere length shortening

Cell culture. The HEK-N/F cells were successively subcultured at a dilution rate of 1:8. The cells were grown to the population doubling level (PDL) of approximately 3 in the absence of the *T. chebula* extract, and fed with or without the extract at a dose of 3 µg/ml successively upon each culture passage. The PDL was regarded to be zero for the culture starting immediately after the primary culture of the HEK-N/F cells, and was calculated to increase according to the following equation: $\log_2$ [(the number of collected cells)/(the number of seeded cells)].

Determination of telomere length by Southern blots. The genomic DNA was extracted using a nucleic acid extraction kit IsoQuick (ORCA Research Inc.) from 10^6 cells at each passage. The extracted DNA was cleaved with the restriction enzyme, *Hinf* I (TaKaRa), to produce terminal restriction fragments (TRFs). The telomere length was determined by Southern blots as previously described (Furumoto *et al*., 1998). Briefly, a portion (2 µg/lane) was loaded onto a 0.8% agarose gel, and electrophoresed at 35 V/cm for 20 h together with a 1 kb DNA Ladder (Gibco BRL) and a lambda DNA/*Hind* III digest (Nippon Gene, Japan) as the size markers. The DNA was depurinated by soaking the gels in 0.2 N NaOH/0.6 M NaCl for 25 min, and transferred to a nitrocellulose membrane Optitran BA-S 85 (Schleicher and Schuel). The DNA was prehybridized with denatured salmon sperm DNA at 50 °C with the oligodeoxynucleotide probe 5′-end [^{32}P]-labeled $(TTAGGG)_4$ (TaKaRa). The membranes were washed with in 4 × SSC/0.1% sodium dodecyl sulfate (SDS) at 55 °C and underwent autoradiography on X-ray film (Scientific imaging film, Kodak), followed by densitometry using a laser densitometer UltraScan XL (Pharmacia).

RESULTS AND DISCUSSION

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

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 *T. chebula* extract induced by *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 *t*-BuOOH

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

Sample treatment (µg/ml)		Cell viability (%)	TBARS (pmole MDA/mg protein)
Control			359.2 ± 65.8**
t-BuOOH		11.2 ± 1.2	9024.5 ± 890.1
+ *T. chebula* extract	50.0	60.5 ± 3.8**	1010.7 ± 117.5**
	25.0	25.1 ± 1.7**	1667.5 ± 201.4**
	12.5	18.9 ± 1.6*	5405.3 ± 297.3*

Values are expressed as mean ± SD of triplicate experiments.
* $P < 0.05$, ** $P < 0.001$, were significantly different from the group treated with *t*-BuOOH.

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/cm^2, the DNA strands of the nucleus was cleaved, which was quantified by the fluorescence intensity of Et_2, 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 administered 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.

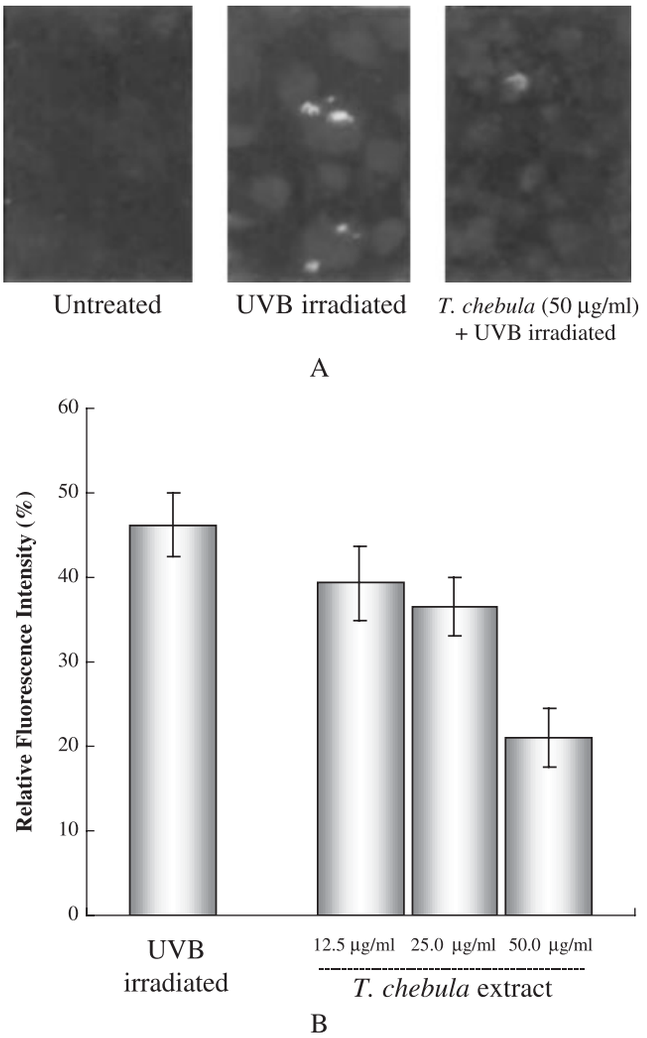


Figure 1. Cytoprotective effect of the *T. chebula* extract on the UVB-induced oxidative stress. The cells were treated with 50 μg/ml of the extract or not for 24 h, rinsed with PBS, and irradiated with UVB. After cultivation for 1–7 h with fresh medium, the cells were stained with 5 μM Et_2. Debris and membrane-disrupted cells were stained with Et_2 as appearing bright in the fluorescent microphotography (A). Relative fluorescence intensity was expressed as percentage of Et_2 fluorescence intensity of tested cells compared to the cells completely destroyed by ethanol (B).

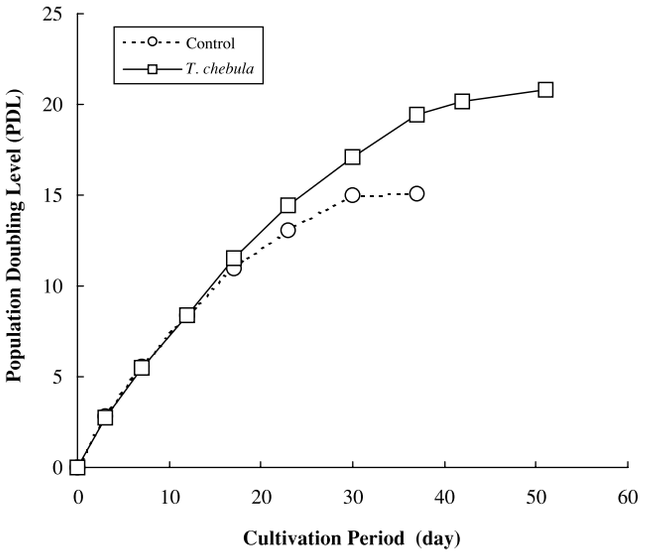


Figure 2. Elongating effect of the *T. chebula* extract on the life-span in the HEK-N/F cells. Cellular life-spans were elongated from PDL 14.9 for control to PDL 20.9 for the *T. chebula* extract-added cells.

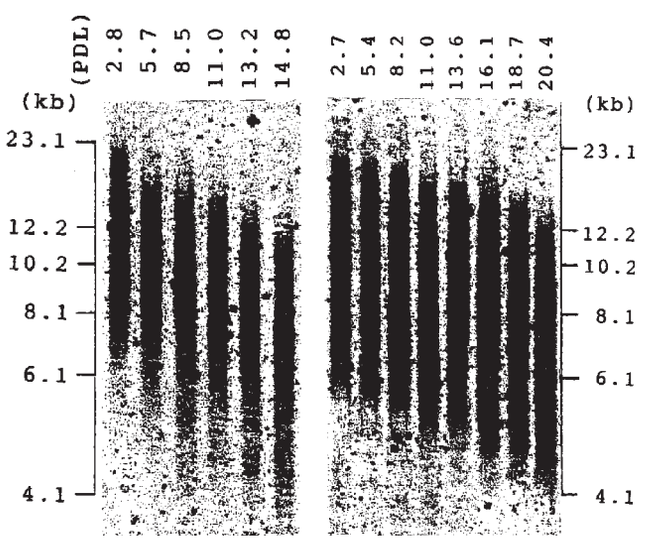
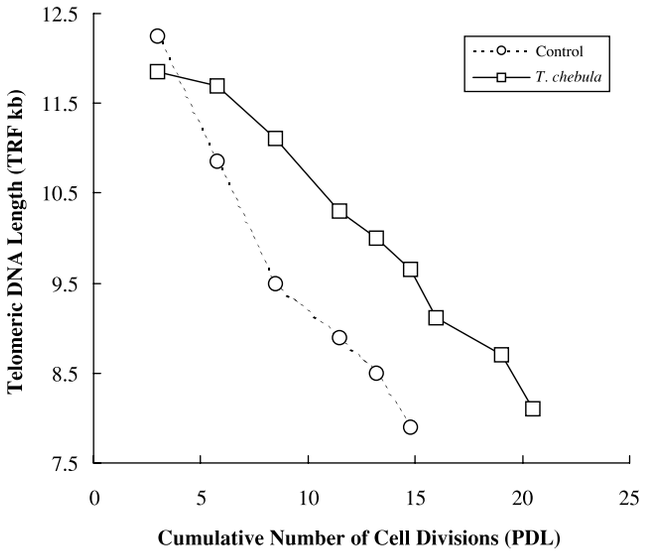


Figure 3. Dependence of the telomere length on the PDLs of the HEK-N/F cells serially subcultivated in the presence or absence of the *T. chebula* extract. The telomere length was determined by Southern blots using a [^{32}P]-labeled $(TTAGGG)_4$ oligonucleotide probe for the TRFs of the genomic DNA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



Figrue 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: $\Sigma(MWi \times ODi)/\Sigma(ODi)$, 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.

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