**Abstract.** The perennial herb, *Humulus japonicus*, has been previously described as possessing potential antituberculosis and anti-inflammatory properties. In the present study, the anti-aging activity of ethanol extracts from the leaves of *H. japonicus* (HJE) was evaluated in yeast and human fibroblast cells. In addition, the antioxidant activity of HJE was analyzed using free radical scavenging assays. Furthermore, the mechanism underlying the hypothesized HJE-associated extension of lifespan was investigated, and the results indicated that HJE was able to extend the lifespan of yeast cells. Further experiments demonstrated that HJE upregulated the longevity-associated proteins, sirtuin 1 and AMP-activated protein kinase, and effectively inhibited the generation of reactive oxygen species (ROS). In addition, the antioxidative potential of the active constituents of HJE, including luteolin, luteolin 7-glycoside, quercetin and quercitrin, was evaluated and the results demonstrated that these flavonoids were able to scavenge ROS in cell-free and intracellular systems. In summary, the results revealed that HJE possessed the potential for antioxidative activity; however, further in vivo investigations are required with the aim of developing safe, high-efficacy anti-aging agents.

**Introduction**

Aging is associated with the deterioration of a number of physiological processes, leading to a decline in functional capabilities, which ultimately impacts the health and overall function of an organism. Among humans and other mammals, these deteriorations occur primarily in the immune system, and result in an increased susceptibility to various conditions, including chronic inflammation, muscle loss, cancer and age-associated degenerative disorders (1). Extensive research over the five previous decades has focused on identifying the underlying mechanisms of aging. In addition to understanding the mechanisms of the aging process, one of the principal aims of research into aging is the identification of intervention strategies or the development of therapeutics that enhance longevity.

It is widely acknowledged that the limitation of calorie intake, also known as calorie restriction (CR), may increase the mean lifespan of an organism by up to 60%, while reducing the incidence of degenerative disease (2). Although the positive effects of CR on slowing the aging process and increasing the lifespan have been demonstrated in a range of species, CR has not been widely adopted as a preventative strategy due to the difficulty of following such a strict dietary regime (3). Therefore, there is a requirement for the identification of small molecules that mimic the effect of CR without the application of a strict diet, or that modulate the molecular pathways responsible for the anti-aging effect produced by CR, in order to slow aging and increase the lifespan of an organism (3). The molecular targets, sirtuin 1 (SIRT1) and AMP-activated protein kinase (AMPK), are potential candidates. SIRT1 is a mammalian ortholog of the yeast protein, silent information regulator 2, and increased activity levels of SIRT1 by activators, such as resveratrol, have been observed to extend the lifespan in a number of species (4-6). Similarly, AMPK has been recognized as a potential molecular target for the regulation of longevity (7). The progression of the aging process has been shown to correlate with a reduction in the activity of SIRT1 and AMPK. Thus, pharmacological interventions aimed at regulating SIRT1 and AMPK may provide effective methods for improving health in aging patients and extending their lifespan (4,5,7).
Medicinal plants have been used for millennia in numerous cultures to prevent and treat a variety of diseases. However, the active constituents of these medicinal plants and their precise mechanisms of action are not fully understood. *Humulus japonicus* Siebold et Zucc, from the Cannabaceae family, is an example of such plants. *H. japonicus* is a perennial herb that grows commonly as a weed in Korea and China, where it is also known as ‘Japanese hop’. In Western countries, *H. japonicus* was previously imported for ornamental purposes; however, the plant is considered to be an invasive plant in numerous countries due to its notable survival capacity. In traditional Chinese medicine, *H. japonicus* has been used to treat pneumonia, diarrhea, hypertension, leprosy and tuberculosis. In Korea, the leaves of *H. japonicus* have been used in the treatment of pulmonary tuberculosis, tuberculosis cervical lymphadenitis and hypertension (8.9). In addition, previous studies have indicated that the extract of *H. japonicus* (HJE) possesses antioxidant, antibacterial, antimycobacterial, antimutagenic, anti-inflammatory and antitumor properties (9-14). Over the previous five decades, a number of the bioactive constituents from *H. japonicus* have been identified and reported, including terpenes, lupulones, phenolics and flavonoids (8,11,15,16).

To the best of our knowledge, the potential of HJE to extend the lifespan and its effect on the aging process have not yet been investigated. Thus, the aim of the present study was to investigate the effect of HJE on lifespan, and to elucidate the signaling pathways and active constituents involved in lifespan extension. In addition, the antioxidant capacities of HJE and its active constituents were evaluated, since reactive oxygen species (ROS) are a major contributing factor to the aging process.

**Materials and methods**

**Chemicals and reagents.** Luteolin, luteolin 7-gluco-side, quercitin, quercitrin and resveratrol were obtained from Sigma-Aldrich (St. Louis, MO, USA), dissolved in ethanol and stored at -20°C until required. In addition, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-morpholinosydnonimine hydrochloride (SIN-1) and carboxy-H$_2$DCFDA were obtained from Sigma-Aldrich. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from GE Healthcare (HyClone; Logan, UT, USA). Rabbit polyclonal antibodies against phospho-AMPKα1/2 (Thr172; cat. no. sc-33524), SIRT1 (cat. no. sc-15404) transcription factor IIB (cat. no. sc-225) and mouse monoclonal β-actin (cat. no. sc-47778), and goat anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated (cat. no. sc-2004) and anti-mouse IgG-HRP-conjugated (cat. no. sc-2031) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 2’,7’-Dichlorodihydrofluorescein diacetate (H$_2$DCFDA) was purchased from Invitrogen Life Technologies (Eugene, OR, USA).

**Plant materials and extraction.** Fresh leaves of Acanthopanax sessiliflorus, Rubus crataegifolius (Bunge), Vitis thunbergii var. sinuata and *H. japonicus* were collected in Busan (Korea) and authenticated by Professor JS Choi at Pukyong National University (Busan, Korea). These plant specimens were deposited in Professor Jae Chung’s labora-
tory (Pusan National University, Busan, Korea). The fresh leaves of these plants were dried, chopped into small pieces and refluxed with absolute ethanol (EtOH). The extract of each plant was separated from the residues through Whatman No. 1 filter paper (GE Healthcare Life Sciences, Pittsburgh, PA, USA), then concentrated to dryness to render the EtOH extract. The extract was subsequently suspended in EtOH and stored at -20°C until required.

**Yeast strain and microbiological methods.** A BY4742 yeast strain (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; EUROSCARF, Frankfurt, Germany) was used for chronological lifespan (CLS) measurements, as described previously (17). Yeast was grown to exponential phase in rich yeast, peptone, dextrose medium or in synthetic defined minimal medium (Sigma-Aldrich), both containing 2% glucose, which were prepared as described by Sherman (18). CLS measurements were performed as previously described (19).

**Inhibition of total ROS generation.** The scavenging activity of the agents under investigation was assessed using H$_2$DCFDA, a fluorescent oxidative stress indicator. For the measurement of ROS-scavenging activity in a cell-free system, H$_2$DCFDA was mixed with esterase (pH 7.4) and incubated for 20 min at 37°C. The mixture was then placed on ice in the dark until immediately prior to measurement. H$_2$DCFDA was hydrolyzed to non-fluorescent 2’,7’-dichlorodihydrofluorescein (DCFH) by esterase (Sigma-Aldrich) and subsequently oxidized to highly fluorescent 2’,7’-dichlorofluorescein by the ROS, O$_2$ (20). The fluorescence intensity of the oxidized DCFH was quantified using a GENios fluorescence microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 485 and 530 nm, respectively. Measurement was performed for 30 min, with or without the addition of SIN-1 as an ·O$_2$ donor. In addition, a similar experiment was performed using Trolox as a positive control to compare for antioxidant capacity.

**Cell culture.** Human fibroblast Hs27 (CRL-1634) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 5% CO$_2$ in air. The fibroblast cells were plated at 90-95% confluency for all the experiments.

**Inhibitory activity on intracellular ROS generation.** Intracellular ROS generation was measured using carboxy-H$_2$DCFDA, a cell-permeable dye. This compound is oxidized intracellularly by ROS to form fluorescent DCF. Briefly, the Hs27 cells were incubated for 24 h in a 96-well plate. After one day, the medium was replaced with fresh serum-free medium containing HJE or flavonoids. The cells were pretreated with HJE or flavonoids for 1 h and were then exposed to ultraviolet B (UVB), according to designated experimental conditions. UVB irradiation was carried out using a UV Crosslinker (CL-1000; UVP, LLC, Upland, CA, USA) at the desired intensity (100 J/m$^2$). Prior to UVB exposure, the cells were washed with phosphate-buffered saline (PBS) and resuspended in fresh PBS. Subsequently, the cells were incubated with 10 µM carboxy-H$_2$DCFDA for 10 min.
at 37˚C, and washed twice with PBS. Modulations in fluorescence intensity were measured every 5 min for 30 min using a GENios fluorescence plate reader, at excitation and emission wavelengths of 485 and 530 nm, respectively.

Cytosolic and nuclear extract preparations. Cells were washed with ice-cold PBS and harvested. A buffer containing 10 mM Tris (pH 8.0), 1.5 mM MgCl₂, 1 mM DTT, 0.1% Nonidet P-40 and protease inhibitors was used to extract the cytosolic fractions by centrifugation at 14,000 x g for 15 min at 4˚C. Nuclear fractions were extracted from the resulting pellets using a buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 100 mM NaCl and protease inhibitors. Aliquots of the cytosolic or nuclear extracts were boiled in gel loading buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 5 min.

Western blot analysis. In order to determine the expression levels of the proteins under investigation, cell extracts were prepared and western blot analysis was conducted. In brief, cell extracts containing equal quantities of proteins (20 µg) were subjected to 8-10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were probed with the primary antibodies (1:1,000 dilutions) overnight at 4˚C, followed by the HRP-conjugated secondary antibodies (1:5,000 dilutions) for 1 h at room temperature. Signals were detected using an enhanced chemiluminescence reagent (AbFrontier Co., Ltd., Seoul, Korea).

Statistical analysis. Analysis of variance was used to analyze the differences between each group, and Dunnett’s multiple comparison test was used to determine the differences between the mean values of the groups. All statistical analyses were conducted using GraphPad Prism version 5.02 (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

HJE extends the yeast lifespan. Effects of HJE on the lifespan of yeast were investigated. In traditional Korean medicine, plants with palmate (hand-shaped) leaves are considered to possess health benefits. Thus, extracts of Acanthopanax sessiliflorus, Humulus japonicus, Vitis thunbergii var. sinuata and Rubus crataegifolius (Bunge) were also examined. To evaluate the anti-aging effect of the plant extracts, yeast cells were cultivated with the extracts for 25 days and the CLS was measured by monitoring the number of colony-forming units (CFUs). The number of viable yeast cells that were able to reproduce and form colonies reduced in culture over time, regardless of the presence of plant extracts. As presented in Fig. 1A, the number of CFUs was higher in the HJE-treated cultures when compared with untreated control cultures between days 10 and 25. However, cultivation with the extracts from A. sessiliflorus, V. thunbergii var. sinuata or...
**R. crataegifolius** (Bunge) was shown to have no effect on the yeast lifespan. Therefore, the HJE exhibited comparatively notable lifespan extension, and the effects of different concentrations of HJE on the yeast lifespan were subsequently examined. The results indicated that HJE increased the viability of yeast cells in a concentration-dependent manner (Fig. 1B). Collectively, these results indicated that HJE exerts a beneficial anti-aging effect.

**HJE modulates the expression levels of AMPK and SIRT1.** A number of previous studies have demonstrated that AMPK and SIRT1 serve important functions in the aging process (21-23). As HJE was observed to exert a beneficial effect on the lifespan of yeast, the effect of HJE on AMPK and SIRT1 expression was subsequently examined. AMPK is the principal energy sensor in eukaryotic cells and functions to maintain cellular energy homeostasis and mitochondrial biogenesis (24). Aging is associated with a reduction in AMPK-induced mitochondrial biogenesis, and the activation of AMPK has been observed to increase the lifespan of fruit flies (25,26). The results of the present study indicated that HJE readily activated AMPK (Fig. 2).

SIRT1 is predominantly located in the nuclei and is responsible for oxidative stress. In addition, a decrease in nuclear SIRT1 levels has been previously reported in the hearts of aged mice (27,28). Therefore, in the present study, nuclear proteins were employed for the detection of SIRT1. The results demonstrated that HJE treatment increased nuclear SIRT1 levels in a concentration-dependent manner (Fig. 2), indicating that HJE may extend the lifespan by modulating the expression levels of AMPK and SIRT1. However, further mechanistic experiments are required at the molecular and organism levels.

**HJE inhibits ROS generation.** The effect of HJE on ROS generation was evaluated to elucidate the mechanism underlying the HJE-mediated extension of yeast lifespan. An equivalent concentration of Trolox, a water-soluble vitamin E analog, was used as a positive control for comparison with the inhibitory effect of HJE on SIN-1-induced ROS in a cell-free system. As presented in Fig. 3A, HJE appeared to scavenge the ROS generated by SIN-1 in a concentration-dependent manner. However, the HJE-induced inhibition of ROS generation was lower compared with that of Trolox.

As the antioxidative capacity of the testing molecules may have differed between cell-free and intracellular systems, the antioxidative effect of HJE was examined in Hs27 human fibroblast cells. UVB radiation has been reported to induce ROS generation, resulting in cellular senescence, and the role of SIRT1 in UVB-induced skin aging is well-established (29,30). Thus, UVB was used to induce ROS generation in the Hs27 skin fibroblast cells. Pretreatment with HJE (5 µg/ml) resulted in a significant reduction (34.8%) in the generation of ROS when compared with the UVB-exposed control cells (Fig. 3B). These results indicated that HJE effectively scavenged ROS in the cell-free system and UVB-stimulated fibroblast cells.

**HJE-derived flavonoids suppress ROS generation.** *H. japonicus* is known to contain a number of flavonoids and phenolics that are responsible for various biological activities (8,11,12). In order to investigate whether the antioxidative capacity of HJE is mediated by the aforementioned active constituents, the effects of luteolin, luteolin 7-glucoside, quercetin and querectin (structures shown in Fig. 4A) on oxidative stress systems, the antioxidative capacity of HJE was evaluated in a cell-free system and UVB-stimulated fibroblast cells. UVB-exposed Hs27 cells were pretreated with HJE (5 µg/ml) for 1 h and further treated with 10 J/m² UVB. The results of the present study indicated that HJE-derived flavonoids suppress ROS generation.

Next, the intracellular antioxidative effect of HJE-derived flavonoids was investigated. UVB radiation was used to induce ROS generation in order to assess the capacities of the HJE-derived flavonoids to inhibit intracellular ROS.
The scavenging activities of the active constituents on UVB-induced ROS in pretreated Hs27 cells are shown in Fig. 5. In particular, luteolin was observed to exert a marked ROS scavenging effect on intracellular ROS, while luteolin 7-glucoside was most effective at scavenging ROS in the cell-free system. Thus, the results indicated clear differences in the antioxidative capacity among the HJE-derived flavonoids. The rank order was as follows: Luteolin > luteolin 7-glucoside = quercetin > quercitrin in the intracellular system. These results clearly demonstrated that the potent antioxidative properties of HJE may be mediated by flavonoids.
Discussion

The present study aimed to investigate the effects of HJE on yeast lifespan. Furthermore, the effect of HJE on the expression levels of SIRT1 and AMPK, which are involved in lifespan modulation, was determined. In addition, as oxidative stress is a major contributing factor in the aging process, the antioxidative capacities of HJE and its active constituents were assessed.

To the best of our knowledge, the present study is the first to propose that HJE may be able to increase the lifespan and delay the detrimental health effects associated with aging. Previous studies have hypothesized that medicinal plants, such as *Lithospermum erythrorhizon*, *Panax ginseng*, *Ginkgo biloba* and *Rhodiola rosea*, may exert beneficial effects on cellular senescence and longevity, which are associated with the aging process (31-34). Although *H. japonicus* has been described and used as a traditional remedy in Korea and China, limited information is available with regard to the underlying biological activities, active phytochemicals and action mechanisms of this plant.

In the present study, HJE was observed to activate AMPK in human fibroblast cells. AMPK has been demonstrated to serve a key function in the process of aging and the determination of lifespan (35). Overexpression of AMPK has been associated with prolonged lifespans in *Caenorhabditis elegans* and the *Drosophila* fruit fly (26,36). In addition, Greer *et al.* demonstrated that the presence of AMPK is essential for lifespan extension by CR in *C. elegans* via phosphorylation of the FOXO transcription factor (37). Notably, AMPK has been reported to phosphorylate FOXO3 in mammalian cells, indicating that the modulation of FOXO by AMPK may be conserved among species (38). Thus, these studies indicate that the activation of AMPK is involved in the extension of lifespan (36,35-38). The results of the present study are consistent with those of previous studies, which have demonstrated that small molecules, such as chioric acid and metformin, are able to prolong lifespan in worms via modulation of AMPK expression (39,40). Furthermore, previous studies have reported that quercetin, quercetin and luteolin, the active constituents of HJE, activate AMPK, which indicates that these active constituents may contribute to the effect of HJE on AMPK expression levels (41-43).

An additional possible mechanism for the life-extending effect of HJE involves SIRT1. The function of sirtuins in lifespan modulation in yeast was recognized over a decade ago; however, the capacity of sirtuins to extend lifespans in other organisms remains controversial. There are seven sirtuin homologs (SIRT1-7) in mammals, of which SIRT1 is the most extensively studied. In mammals, the anti-aging mechanism underlying CR has been shown to involve the activation of SIRT1 in numerous tissues (44). Thus, increased expression of SIRT1 in mice results in phenotypes that resemble the lifespan-extending effects of CR (45). Since SIRT1 performs a key function in lifespan modulation, the protein has attracted increasing attention as a potential drug target for delaying the onset of aging and extending the lifespan. For example, the polyphenol, resveratrol, which has been identified in red wine and grapes, targets SIRT1 and exerts a beneficial effect on lifespan (5,6). Furthermore, reduced levels of SIRT1 have been observed in aged mouse heart tissue (28). Therefore, it is possible that the lifespan-extending effect of HJE is mediated by SIRT1 regulation. However, the effect exerted by HJE on the lifespans of higher order organisms is yet to be fully elucidated.

A recognized mechanism underlying the aging process is the accumulation of oxidative damage; a hypothesis that has been widely accepted (46). Thus, an antioxidative effect may result in lifespan extension. For example, the ability of resveratrol to function as an antioxidant is a possible alternative mechanism for its lifespan-extending effect, other than the activation of sirtuins (47). Results from the present study and previous studies indicate that HJE and its active flavonoid constituents exhibit antioxidative activity (10,12,13). Notably, a discrepancy in the antioxidative activity of these active constituents was observed between cell-free and cell culture systems. The aglycone forms of the flavonoids (luteolin and quercetin) exerted more potent ROS scavenging activities in Hes27 cells when compared with their sugar-conjugated forms (luteolin 7-glucoside and quercitrin). The results of the previous study demonstrated that the antioxidative activity of flavonoids is determined by the position, number and state of the hydroxyl groups located on the benzene ring, and that the glycosylation of these hydroxyl groups results in a reduction in antioxidative capacity (48). Thus, the role of the sugar moiety in the antioxidative activity of flavonoids remains controversial, and depends on the type and location of the sugar group. Although luteolin exerted the strongest ROS scavenging capacity, quercetin is the most extensively studied molecule among the active constituents of *H. japonicus*. Cheng *et al.* reported that the antioxidative activity of quercetin in *C. elegans* was enhanced if the molecule was sugar-conjugated (49). Furthermore, the authors proposed that the antioxidant effect may be attributable to the moiety promoted by the rhamnopyranoside, which is able to facilitate flavonoid absorption, as described in the animal model (49,50). However, Comalada *et al.* reported that the anti-inflammatory effect exerted by quercetin appeared to be mediated by the release of quercetin, which was generated by glycoside cleavage in rat intestinal microbiota (51). In accordance with these observations, Jiang *et al.* demonstrated that quercetin is a major metabolite of quercitin, and that the production of quercetin was able to improve the absorption rate and bioavailability of quercitin in *vivo* (52). These results may explain the finding that aglycone forms of HJE-derived flavonoids exhibit stronger antioxidative capacities compared with those of the sugar-conjugated forms in a cell culture system.

In conclusion, the results obtained in the present study demonstrated that pretreatment with HJE enhanced the lifespan of yeast. Furthermore, HJE was shown to exert antioxidative activities in a cell-free system and in human fibroblast cells. In addition, the active constituents of *H. japonicus*, namely luteolin, luteolin 7-glucoside, quercetin and quercitrin, exhibited antioxidative capacities, with luteolin exerting the most notable ROS scavenging activity among the tested constituents. Thus, the results of the present study indicate that HJE may have the potential to be used as a source for the development of pharmacological or nutraceutical interventions that delay the aging process and extend longevity. However, further studies in animals and humans are required to fully determine the potential of this medicinal plant for improving human health.
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