

Evaluation of *in vitro* anti-oxidant and anti-inflammatory activities of Korean and Chinese *Lonicera caerulea*

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BACKGROUND/OBJECTIVE: The honeysuckle berry (HB) contains ascorbic acid and phenolic components, especially anthocyanins, flavonoids, and low-molecular-weight phenolic acids. In order to examine the potential of HB as a hepatoprotective medicinal food, we evaluated the *in vitro* anti-oxidant and anti-inflammatory activities of Korean HB (HBK) and Chinese HB (HBC).

MATERIALS/METHODS: Antioxidant and anti-inflammatory effects of the extracts were examined in HepG2 and RAW 264.7 cells, respectively. The anti-oxidant capacity was determined by DPPH, SOD, CAT, and ARE luciferase activities. The production of nitric oxide (NO) as an inflammatory marker was also evaluated. The *Nrf2*-mediated mRNA levels of heme oxygenase-1 (*HO-1*), NAD(P)H dehydrogenase [quinone] 1 (*Nqo1*), and glutamate-cysteine ligase catalytic subunit (*Gclc*) were measured. The concentrations of HB extracts used were 3, 10, 30, 100, and 300 µg/mL.

RESULTS: The radical scavenging activity of all HB extracts increased in a concentration-dependent manner ($P < 0.01$ or $P < 0.05$). SOD ($P < 0.05$) and CAT ($P < 0.01$) activities were increased by treatment with 300 µg/mL of each HB extract, when compared to those in the control. NO production was observed in cells pretreated with 100 or 300 µg/mL of HBC and HBK ($P < 0.01$). Treatment with 300 µg/mL of HBC significantly increased *Nqo1* ($P < 0.01$) and *Gclc* ($P < 0.05$) mRNA levels compared to those in the control. Treatment with 300 µg/mL of HBK ($P < 0.05$) and HBC ($P < 0.01$) also significantly increased the *HO-1* mRNA level compared to that in the control.

CONCLUSIONS: Thus, the Korean and Chinese HBs were found to possess favorable *in vitro* anti-oxidant and anti-inflammatory activities. *Nrf2* and its related anti-oxidant genes were associated with both anti-oxidant and anti-inflammatory activities in HB-treated cells. Further studies are needed to confirm these *in vivo* effects.

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INTRODUCTION

The liver plays vital roles in several processes, including protein synthesis, glucose homeostasis, detoxification, and cycling of various nutrients [1,2]. Liver damage causes metabolic dysfunctions. If liver injury persists, transient elevation of liver enzymes, hepatic fibrosis, cirrhosis, and hepatocellular carcinoma occur sequentially or simultaneously [3]. Oxidative stress and inflammation are closely related to the etiology of liver damage [4]. Although treatments are continuously being developed to protect the liver from damage, reliable hepatoprotective drugs remain insufficient. Therefore, it is necessary to develop

functional foods that can help prevent and treat liver damage.

Oxidative stress is induced by reactive oxygen species (ROS) that can damage cells and result in diseases such as diabetes, cardiovascular ailments, cancer, and liver damage [5]. Anti-oxidant vitamins and enzymes, such as superoxide dismutase, glutathione peroxidase, and glutathione reductase, can reduce excessive ROS production [6]. Phytochemicals with distinct flavors, odors, and colors can also exhibit anti-oxidant activities, and it has been reported that berries contain many phytochemicals such as a flavonoids, anthocyanins, and phenolic acids [7].

The honeysuckle berry (HB) *Lonicera caeruleae* has been used

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in folk medicine in China, Japan, and northern Russia. However, the HB is not well-known as an edible berry in North America, Europe, or Korea. HB contains ascorbic acid and a number of phenolic components, especially anthocyanins, flavonoids, and low molecular-weight phenolic acids. It has been reported that HB extract exerts strong anti-oxidant effects [8], radioprotective effects against ionizing radiation in mice [5], hepatoprotective effects [9], anti-inflammatory effects [10,11], improvement of lipid and glucose metabolism [12], and therapeutic effects on hyperthyroidism [13]. Especially, HB extract has been shown to have the strongest anti-oxidant activity among 12 types of colored berries [14]. Abundant phenols in HB extract have been shown to possess anti-inflammatory and wound-healing properties both *in vitro* and *in vivo* [10]. Furthermore, they have shown skin-protective effects against ultraviolet-induced damages [15,16].

Nuclear factor-erythroid 2-related factor-2 (*Nrf2*) induces the expression of anti-oxidant-related genes such as *HO-1*, *Nqo1*, and *Gclc*, and these genes were reported to reduce proinflammatory cytokine levels [17,18]. Furthermore, it has been indicated that proinflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukin (IL)-6, affect lipopolysaccharide (LPS)-stimulated production of nitric oxide (NO) [19,20]. Although a number of studies with respect to anti-oxidant and anti-inflammatory effects of the HB have been reported, studies regarding the expression of *Nrf2*-related anti-oxidant genes by HB extracts have been scarce.

Recently, HB cultivation in Korea has been increasing, although HB used in the Korean industry is mainly imported from China. HB has attracted attention as a health food, but has not been developed as a functional food for improving liver function. Therefore, in order to examine the possibility of using HB as a hepatoprotective medicinal food, we evaluated the *in vitro* anti-oxidant and anti-inflammatory efficacy, luciferase reporter activity, and *Nrf2*-related gene expression of Korean and Chinese HB (HBK and HBC, respectively).

MATERIALS and METHODS

Cell culture

The human hepatocyte cell line (HepG2) and murine macrophage cell line (RAW 264.7) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were seeded in 6-well plates (1×10^5 per well) at 70-80% confluence. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco[®], MA, USA) containing 10% fetal bovine serum (Hyclone[™], Victoria, Australia), 50 units/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 50 μ g/mL streptomycin (Sigma-Aldrich) in a 5% CO₂ humidified incubator at 37°C.

Test materials

HBK was prepared and supplied by Aribio Co. Ltd. (Seongnam, Korea). HBC was imported from China and supplied by H&K Bioscience Co., Ltd. (Seoul, Korea). Briefly, HBK was prepared as follows: HBK samples were squeezed, decompressed, and condensed at 55-65°C (EYELA, N-N series, Tokyo, Japan) and completely lyophilized in a freeze dryer (Operon FDB-5503, Kimpo, Korea). For HBC preparation, concentrated Chinese HB

juice (63 brix) was decompressed and condensed and then lyophilized as specified previously for HBK. The prepared samples were stored at -20°C until further use. The concentrations of HB extracts used were 3, 10, 30, 100, and 300 μ g/mL.

Radical scavenging activity assay

Radical scavenging activity was determined using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay [21]. Briefly, DPPH (Sigma-Aldrich) in ethanol solution (150 μ M) was incubated with HBK (10-300 μ g/mL), HBC (10-300 μ g/mL), or Trolox (100 μ M), an anti-oxidant derived from α -tocopherol. Samples were incubated at room temperature for 30 min under light protection. The absorbance was measured at a wavelength of 517 nm using a microplate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland).

Cell viability assay

HepG2 or RAW 264.7 cells were cultured in 24-well plates (5×10^4 cells/well). To examine the cytoprotective effect of each HB extract in HepG2 cells, 3-300 μ g/mL of each HB extract was pre-incubated with HepG2 cells at 1 h prior to the addition of 150 μ M tert-butyl hydroperoxide (*t*-BHP) for 12 h. Likewise, in RAW 264.7 cells, 3-300 μ g/mL of each HB extract was pre-incubated with cells at 1 h prior to the addition of 1 μ g/mL LPS for 18 h. After incubation, viable cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.1 μ g/mL) for 4 h.

Reporter gene assay

To determine luciferase activity, stably transfected cells with pGL4.37 were seeded onto 12-well cell culture plates at a density of 5×10^5 cells/well followed by serum starvation for 12 h. Cells were then exposed to 10-300 μ g/ml of each HB extract for 24 h. After adding a luciferase assay reagent (Promega), luciferase activities in cell lysates were measured using a microplate reader (Tecan).

Total RNA isolation and Real-time PCR

Total RNA was isolated from treated cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA (2 μ g each) was then reverse-transcribed to cDNA using oligo-d(T)16 primers on a thermal cycler (Mastercycler Nexus Gradient Thermal Cycler, Eppendorf AG, Hamburg, Germany). PCR was performed using gene-specific primers targeting human heme oxygenase-1 (*HO-1*) (sense, CAGGAGCTGCTGACCCATGA; antisense, AGCAACTGTCG CCACCAGAA; PCR product size, 195 bp), glutamate-cysteine ligase catalytic subunit (*Gclc*) (sense, GAAGTGGATGTGGACA CCAG; antisense, TTGTAGTCAGGATGGTTTGCGA; PCR product size, 128 bp), NAD(P)H dehydrogenase quinone 1 (*Nqo1*) (sense, GGATTGGACCGAGCTGGAA; antisense, TGCAGTGAAGATGAAGG CAAC; PCR product size, 137 bp). Primers were synthesized by Bioneer (Daejeon, Korea).

Superoxide dismutase (SOD) and catalase (CAT) activity assay

SOD and CAT activities were determined by using HepG2 cell lysates after cells were treated with 300 μ g/mL of each HB extract for 24 h. Catalase activity in cell homogenate was measured by using a catalase assay kit (Cayman Chemical, Ann Arbor, MI, USA). The activity of SOD in cell homogenate was

determined using a superoxide dismutase assay kit (Cayman Chemical). A tetrazolium salt was provided in the kit to detect superoxide radicals generated by xanthine oxidase and hypoxanthine at a wavelength of 550 nm.

Determination of NO production

To quantify NO in the conditioned medium, RAW 264.7 cells were pretreated with 10-300 µg/mL of each HB extract for 1 h and subsequently exposed to 1 µg/mL LPS for 18 h. After treatment, 100 µL of collected culture medium was incubated with an equal volume of Griess reagent. The absorbance was measured at a wavelength of 550 nm by using an automated microplate reader (Tecan). Recorded values are presented as fold versus control.

Statistical analysis

All data are expressed as Mean±SD from three experiments. Multiple comparison tests were performed for different groups according to dose. An equilibrium test was performed using the Levene test. Least-significant differences (LSD) multiple comparisons were used for group comparisons. In cases of unequal distribution, the Kruskal-Wallis H test was performed with Levene test and nonparametric test.

When the Kruskal-Wallis H test showed significant differences, Dunnett's tests were performed to determine specific pairs between groups. Differences were considered significant at $P < 0.05$ level. All statistical analyses were performed using SPSS for Windows (14.0K, SPSS Inc., Chicago, IL, USA).

RESULTS

Radical scavenging activity of HB extract

To compare the *in vitro* anti-oxidant capacity of HB extract of different origins, radical scavenging activity was examined using a DPPH assay. The radical scavenging activity of all HB extracts increased in a concentration-dependent manner (Fig. 1). There were significant differences in radical scavenging activity for both HBK and HBC compared to the control ($P < 0.01$). At a concentration of 100 µg/mL, and HBK and HBC had 33.9% and 42.75% activity, respectively, whereas at 300 µg/mL, they exhibited 86.1% and 92.96% activity, respectively.

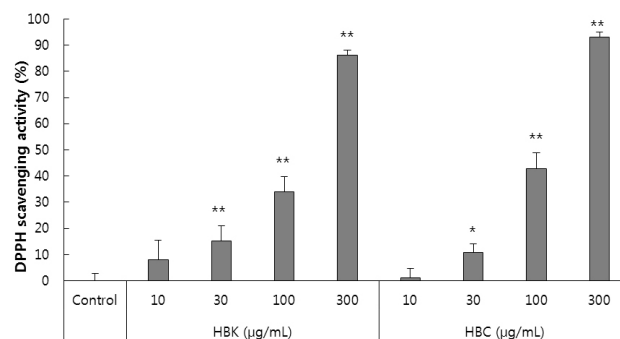


Fig. 1. DPPH scavenging activities of honeysuckle berry extracts. DPPH, 2, 2-Diphenyl-1-Picrylhydrazyl; LSD, least-significant differences; HBK, Korean honeysuckle berry; HBC, Chinese honeysuckle berry. Values are expressed as Mean±SD. Significant compared to the control (100 µM Trolox-treated) by LSD test, ** $P < 0.01$; * $P < 0.05$.

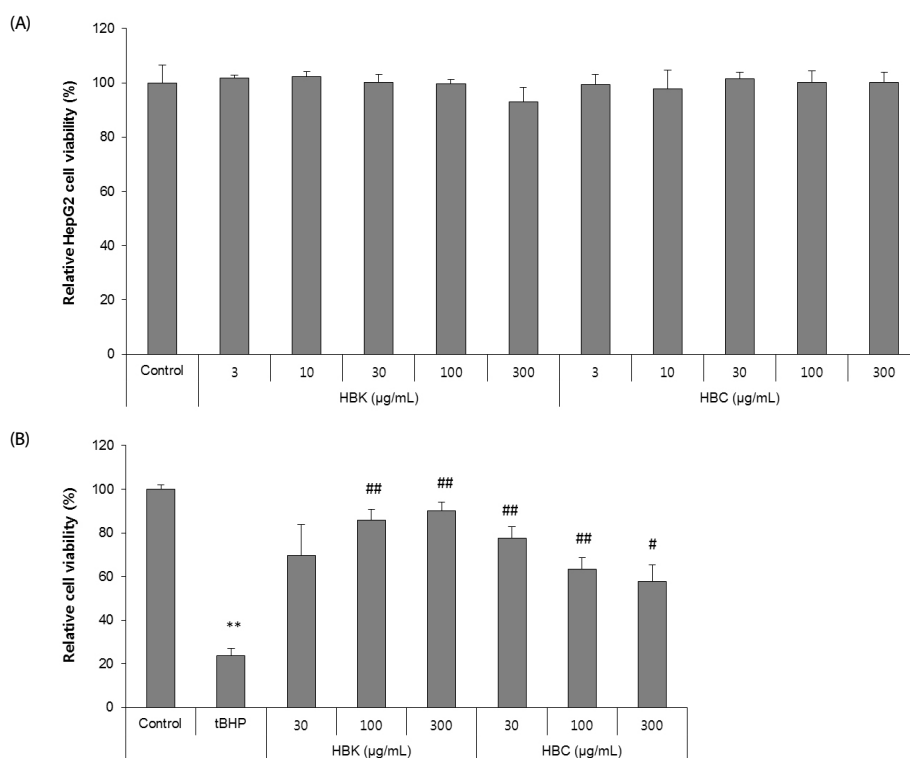


Fig. 2. Effect of honeysuckle berry extract on the viability of HepG2 cells. (A) Cell viability by MTT assay. (B) Cell viability induced by t-BHP. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; t-BHP, *tert*-butyl hydroperoxides; HBK, Korean honeysuckle berry; HBC, Chinese honeysuckle berry. Values were expressed as Mean±SD. Significant compared to the control by Dunnett's test, ** $P < 0.01$ compared to t-BHP treated cells by Dunnett's test; ## $P < 0.01$; # $P < 0.05$.

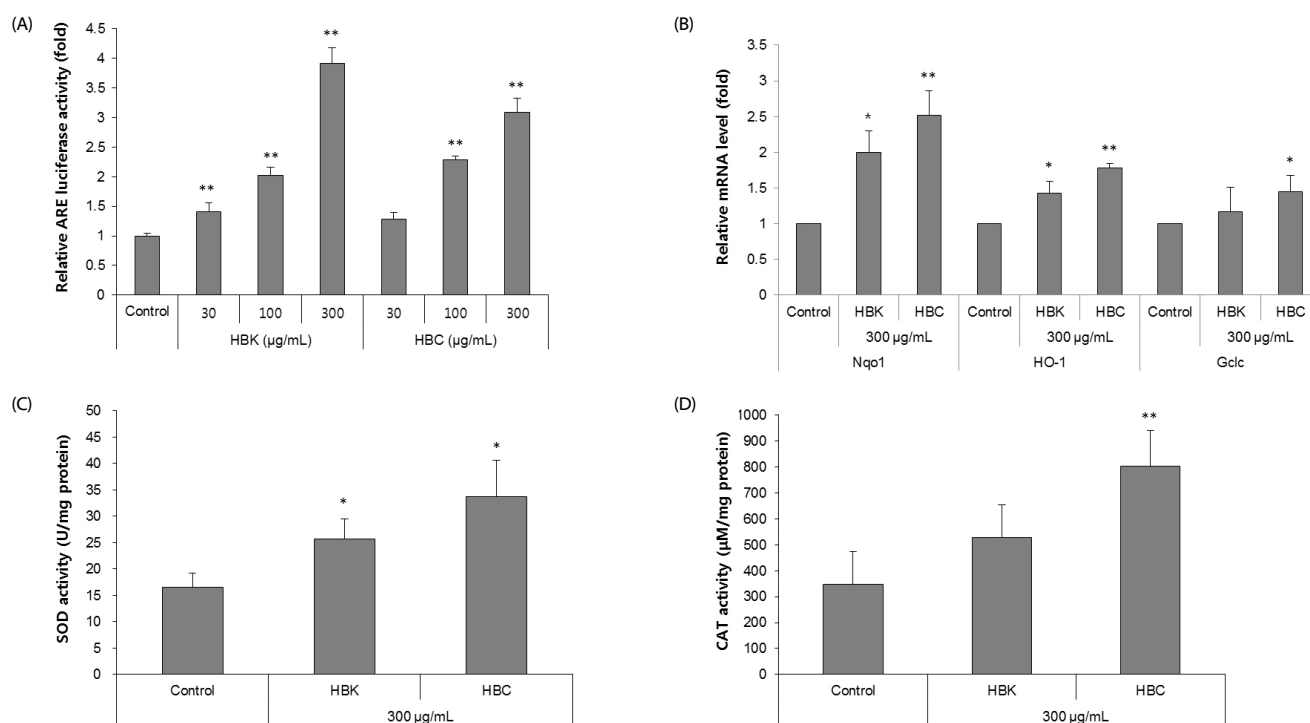


Fig. 3. Effect of honeysuckle berry extract in HepG2 cells. (A) *Nrf2* transactivation, (B) *Nrf2*-dependent anti-oxidant gene expressions, (C) SOD activity, (D) CAT activity. HBK, Korean honeysuckle berry; HBC, Chinese honeysuckle berry; *Nrf2*, nuclear factor (erythroid-derived 2)-like 2; SOD, superoxide dismutase; CAT, catalase. Values are expressed as Mean \pm SD. Significant compared to the control by least-significant differences (LSD) test. ** $P < 0.01$; * $P < 0.05$.

Effect of HB extract on HepG2 cell viability

Cell viability was investigated in HepG2 cells after treatment with 3-300 μ M of each HB extract for 24 h. The results of the MTT assays indicated that treatment with each HB extract up to 300 μ M did not affect the viabilities of HepG2 cells (Fig. 2A). Because 300 μ M of each HB extract did not show any cytotoxicity, this concentration was chosen as the maximal concentration for each HB extract for subsequent experiments using HepG2 cells.

Effect of HB extract on *t*-BHP-induced cytotoxicity

To examine the effect of each HB extract against oxidative stress-mediated cytotoxicity, HepG2 cells were pretreated with 30-300 μ M of each HB extract for 1 h and subsequently exposed to 150 μ M of *t*-BHP for 12 h. Cell viability was then determined by the MTT assay. Exposure to *t*-BHP for 12 h significantly decreased cell viabilities. However, pretreatment with each HB extract increased cell viabilities. Statistically significant differences in cell viabilities were observed between HB pretreated cells and cells treated with *t*-BHP alone (23.6%) compared to the control (Fig. 2B). When an equal concentration of each HB extract used for the pretreatment was compared, HBK at 300 μ M showed the most potent cytoprotective activity (89.99%) against *t*-BHP-induced cytotoxicity. However, differences in cell viabilities were not statistically significant between HBK and HBC-treated cells.

Effect of HB extract on *Nrf2* transactivation

To examine whether *Nrf2* was involved in HB-mediated

cytoprotection, HepG2 cells stably transfected with pGL4.37 containing an anti-oxidant response element (ARE)-driven reporter gene were treated with 30-300 μ M of each HB extract for 24 h, and luciferase activities were then monitored. The treatment with each HB extract significantly increased ARE-driven luciferase activities in a dose-dependent manner. Statistically significant differences in ARE-driven luciferase activities were observed for all HB pretreated cells (except cells treated with 30 μ M of HBC) when compared to those of controls (Fig. 3A). When an equal concentration of each HB extract was compared, HBK (3.9 fold) at 300 μ M showed the most potent induction of luciferase activity.

Effect of HB extract on the expression of *Nrf2*-dependent anti-oxidant genes

To examine whether *Nrf2* activation induced by the HB extract might affect the transcription of anti-oxidant genes, HepG2 cells were treated with 300 μ M of each HB extract for 24 h, and the mRNA levels of anti-oxidant genes were then monitored by real-time PCR. The results of real-time PCR indicated that treatment with each HB extract tended to increase the mRNA levels of *Nqo1*, *HO-1*, and *Gclc*. Treatment with both 300 μ M of HBK and HBC increased mRNA expression, i.e. 1.4-fold and 1.8-fold, respectively, for *HO-1*, and 2.0-fold and 2.5-fold for *Nqo1*, respectively, compared to the control. As for *Gclc*, HBC showed higher mRNA expression than the control (Fig. 3B).

Effect of HB extract on SOD and CAT activities

SOD activities were significantly increased by treatment with

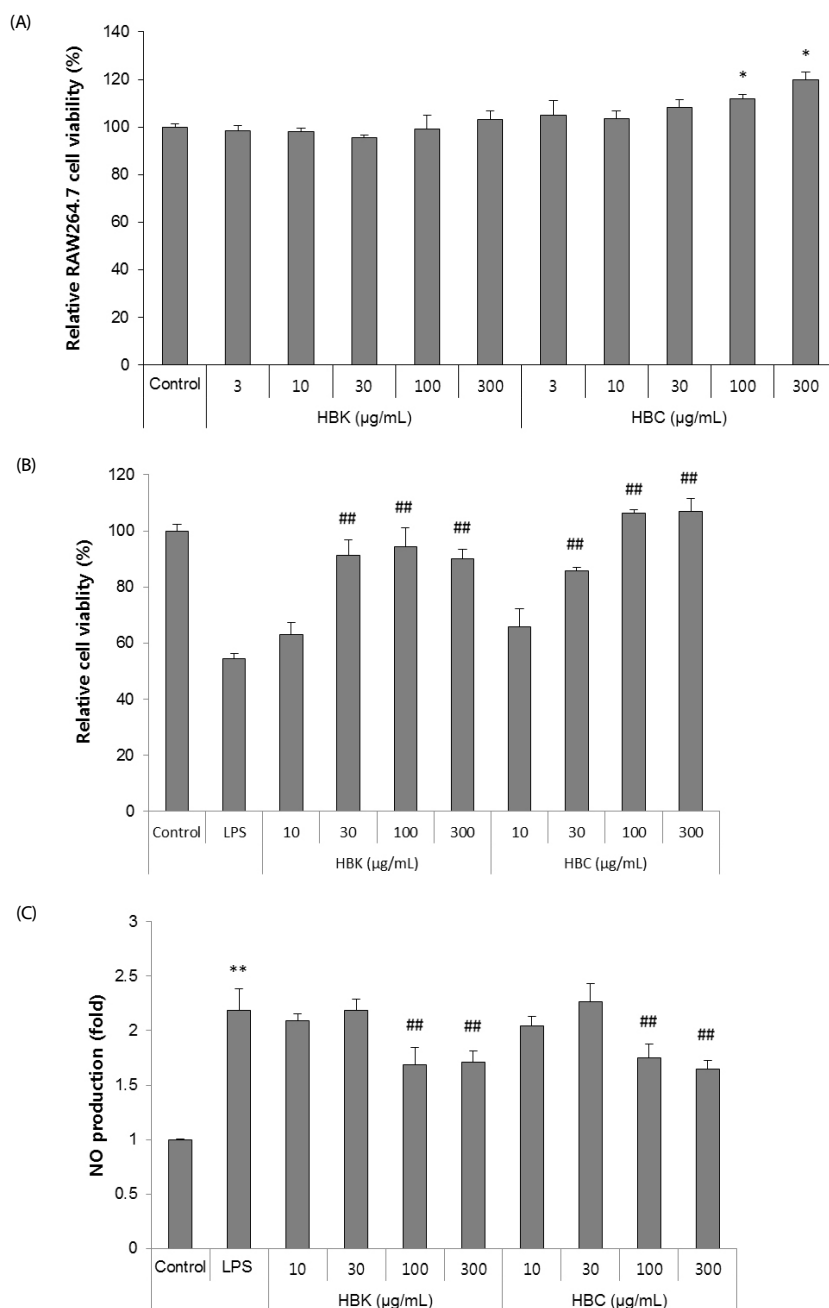


Fig. 4. Effect of honeysuckle berry extract in RAW 264.7 cells. (A) Cell viability by MTT assay. (B) Lipopolysaccharides (LPS)-mediated cell viability. (C) LPS-mediated NO production. HBK, Korean honeysuckle berry; HBC, Chinese honeysuckle berry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide. Values are expressed as Mean±SD. Significant compared to the control by Dunnett's test, * $P < 0,05$; ** $P < 0,01$ compared to LPS-treated cells by Dunnett's test; ## $P < 0,01$.

300 µg/mL of each HB extract (HBK, 25.7 U/mg protein; HBC, 33.7 U/mg protein) as compared to those of the control (Fig. 3C). CAT activities also tended to be increased by treatment with 300 µg/mL of each HB extract (HBK, 506.5 µm/mg protein; HBC, 802.2 µm/mg protein) as compared to those of the control (Fig. 3D). However, statistically significant differences in CAT activities were only observed in 300 µg/mL of HBC-treated cells.

Effect of HB extract on RAW 264.7 cell viability, LPS-induced NO production, and cytotoxicity

Treatment with HBK up to 300 µg/mL did not affect the viabilities of RAW 264.7 cells. However, treatment with 100 or 300 µg/mL HBC significantly increased cell viabilities (Fig. 4A). Statistically significant differences in cell viabilities were observed between HBC and HBK pretreated cells and cells treated with LPS alone (Fig. 4B). When different concentrations of each HB used for the pretreatment were compared, HBC at

300 µg/mL showed the most potent cytoprotective activity against LPS in RAW 264.7 cells. Statistically significant differences in NO production were observed in cells pretreated with 100 and 300 µg/mL, respectively, of HBK and HBC compared to that seen with LPS treatment alone (Fig. 4C).

DISCUSSION

The present study revealed that HB extracts from both origins increased radical scavenging activity in a concentration-dependent manner. Our results are similar to those of a previous study showing that the HB extracts possess the highest anti-oxidant effects among 12 types of colored berries [14]. In our study, HBC at 300 µg/mL showed the most potent radical scavenging activity among all concentrations tested. It was higher than the radical scavenging activity of HBK at the same concentration. Moreover, the results of the MTT assays suggested that each HB extract up to 300 µg/mL had no negative effect on the viabilities of HepG2 or RAW 264.7 cells. Thus, HB extracts of both origins seem to have potent radical scavenging activity without any cytotoxicity. However, further studies are required to evaluate their *in vivo* toxicities.

To explore whether the radical scavenging activity of HB extract contributed to cell protection against oxidative stress, cell viability was monitored after treatment of t-BHP and LPS in HepG2 and RAW 264.7 cells, respectively. As expected, treatment with 150 µM of t-BHP in HepG2 cells and 1 µg/mL of LPS in RAW 264.7 cells significantly decreased cell viabilities. Nevertheless, the pretreatment with each HB extract in HepG2 cells increased cell viabilities. Significant differences in cell viabilities were observed across all HB-pretreated cells (except cells pretreated with 30 µg/mL of HBK) and those treated by t-BHP alone. These results indicated that polyphenol components of the HB extracts have an effect on the cell viability in HepG2 cells induced by t-BHP. This finding is supported by another study where phenolic compounds displayed protective effects against oxidative stress, leading to the maintenance of a normal redox state of the cell [22]. HBK and HBC showed increased cell viabilities in a dose-dependent manner, although 30 µg/mL of HBK did not show a significant increase in cell viabilities. The differences between HBK and HBC cell viabilities may have been due to differences between the geographical location and soil environments of the HBs. The plant's phytochemical content may vary in the composition depending on the environment [23,24]. Also, the HBs used in this study were not representative of their respective sources. Thus, further studies are needed with multiple samples from China and Korea. Both HBK and HBC provided protection by increasing cell viability against LPS-induced cytotoxicity. Statistically significant differences in such protection were observed in all HB-pretreated cells when compared to cells treated with LPS alone. Collectively, these results suggest that HBK and HBC have a potent protective effect on hepatocytes against oxidative stress and inflammation in RAW 264.7 cells in this study.

Phenolic compounds and flavonoids are well-known *Nrf2* inducers [25], and the presence of these compounds in HB extract is expected to activate *Nrf2*. In fact, the highest activity of *Nrf2* was observed at the highest dose of HB. The activation

of *Nrf2* was linked to various cellular signaling pathways such as p38 MAPK, extracellular-regulated protein kinase, phosphatidylinositol 3 kinase, protein kinase C, and casein kinase [26-30]. Therefore, further studies are needed to elucidate the essential components of upstream signaling molecules that contribute to *Nrf2* activation in HB.

Similar to other studies [17,31], treatment with HB extract tended to increase mRNA levels of *Nqo1*, *HO-1*, and *Gclc*. It has been well known that anti-oxidant genes such as *HO-1*, *Nqo1*, and *Gclc* contain AREs in their promoter region, which can be induced by *Nrf2* activation [26,32,33]. Increasing evidence suggests that induction of *HO-1* can protect tissues against a variety of chronic diseases by preventing oxidative stress-mediated apoptosis and inflammation [34]. This is also strongly supported by the observation that the cytotoxic effects of oxidative stress are exacerbated in cells lacking *HO-1* [35,36]. *Gclc* is a heterodimeric enzyme involved in the first rate-limiting regulatory step of glutathione biogenesis. Thus, *Gclc* plays an essential role in maintaining cellular redox homeostasis and reducing oxidative damage by glutathione synthesis [37]. *Nqo1* is a member of the NAD(P)H dehydrogenase family. It involves two-electron reduction of quinones to hydroquinones. Therefore, *Nqo1* could play an important role in preventing the production of ROS [38]. Our study suggests that the compounds of HB extracts have a potential to be developed as hepatoprotective agents since pretreatment with HBK and HBC increased *Nrf2*-mediated anti-oxidant genes.

Anti-oxidant enzymes such as CAT and SOD were also essential in both scavenging ROS and maintaining cellular integrity [39]. From parallel results of real-time PCR, at 300 µg/mL of HBK and HBC, significant increases in SOD and CAT activities were observed. These results were similar to other studies using phenolic-rich plants with elevated SOD and CAT activities [40]. Taken together, HBC and HBK might have a potent effect in inducing anti-oxidant genes, because all HB extracts were capable of transactivating *Nrf2*. An inflammatory response is mainly regulated by NO, eicosanoids, and cytokines, all of which are released by injured cells. They play important roles in the progression of inflammatory states such as edema, intra/intercellular stress, and tissue necrosis. Although NO production under physiological conditions by inflammatory mediators contribute to the elimination of ingested pathogens in macrophages, excessive and prolonged production of NO may attack neighboring cells through reactive nitrogen species, which can accelerate inflammation-mediated tissue injuries.

Because common cellular signaling pathways are involved in the production of proinflammatory mediators in macrophages, monitoring changes in NO production in macrophages is a representative method to examine the anti-inflammatory potential of drug candidates. Therefore, instead of HepG2 cells, we measured NO production using RAW 264.7 cells, which is a kind of macrophage. Pretreatment with HBK and HBC significantly decreased NO production at a high dose compared to that at a low dose. Previous studies reported that the enhanced expression of ROS scavengers, such as *Nqo1* and *Gclc*, has beneficial effects on improving immunity [17-20]. The present study revealed elevated expressions of *Nrf2*-mediated anti-oxidant genes and reduced NO production at 300 µg/mL

of HB. Further studies are needed to explore the effects of HB on other pro-inflammatory mediators and to elucidate the signaling pathways involved.

In conclusion, this study showed that HB extract has favorable *in vitro* anti-oxidant and anti-inflammation activities, and *Nrf2* and its related anti-oxidant genes were associated with both anti-oxidant and anti-inflammatory effects in HB-treated cells. Our results suggest that both HBK and HBC are potentially suitable as preventive and therapeutic foods.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

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