



## Single Oral Dose Toxicity Test of Blue Honeysuckle Concentrate in Mice

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The objective of this study was to obtain single oral dose toxicity information for concentrated and lyophilized powder of blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae; BHcL) in female and male ICR mice to aid in the process of developing natural origin medicinal ingredients or foods following proximate analysis and phytochemical profile measurement. The proximate analysis revealed that BHcL had an energy value of 3.80 kcal/g and contained 0.93 g/g of carbohydrate, 0.41 g/g of sugar, 0.02 g/g of protein, and 0.20 mg/g of sodium. BHcL did not contain lipids, including saturated lipids, trans fats, or cholesterol. Further, BHcL contained 4.54% of betaine, 210.63 mg/g of total phenols, 159.30 mg/g of total flavonoids, and 133.57 mg/g of total anthocyanins. Following administration of a single oral BHcL treatment, there were no treatment-related mortalities, changes in body weight (bw) or organ weight, clinical signs, necropsy or histopathological findings up to 2,000 mg/kg bw, the limited dosage for rodents of both sexes. We concluded that BHcL is a practically non-toxic material in toxicity potency.

**Key words:** Single oral dose toxicity, Mouse, Blue honeysuckle, Proximate analysis, Phytochemical analysis

### INTRODUCTION

Medicinal plants, herbs, and some crude drug substances may be potential antioxidant sources useful in combating various diseases. Therefore, these compounds are important to the pharmaceutical industry and have inspired searches for new sources of bioactive molecules (1,2). Demand and consumption of functional foods that originate from natural sources has increased recently due to the increased interest in functional foods and their contribution to general well-being (3). However, the toxicological aspects of these naturally originating functional foods have been neglected because they have been used for various

purposes for a long time (4). Therefore, more detailed and systemic toxicological studies are necessary to control abuse and potential toxicity, even if the compounds have been routinely used in traditional folk medicine (5).

Blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae; BH) is a traditional shrub used in folk medicine in northern Russia, China, and Japan; its fruit is known as edible berries in North America, Europe, and Korea (6). The berries are a rich source of ascorbic acid and phenolic components, particularly anthocyanins, flavonoids, and low-molecular-weight phenolic acids (6,7). These compounds reportedly have multiple biological activities, including strong antioxidant activity (6). A phenolic-rich extract of BH reportedly exerted anti-inflammatory and wound-healing effects *in vitro* and *in vivo* (8). Recently, orally administered BH reportedly protected mice against ionizing radiation (9), reduced ultraviolet (UV)-induced damage (10), ameliorated abnormal lipid and glucose metabolism in rats (11), and exerted hepatoprotective (12) and anti-inflammatory effects (13). BH had the most potent antioxidant activity among 12 types of colored berries (14). However, no detailed toxicological assessments of BH exist; even the most basic rodent single oral dose toxicity tests have not

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been performed.

In the present study, a single oral dose toxicity test of concentrated and lyophilized powder of BH (BHcL) was conducted in female and male ICR mice according to the Korea Food and Drug Administration (KFDA) Guidelines (15). We aimed to obtain primary safety information and further clarify its safety for clinical use. Proximate and phytochemical analyses revealed that BHcL contained betaine, total phenols, flavonoids, and anthocyanins.

## MATERIALS AND METHODS

**Preparation of BHcL.** A deep purple colored solution of concentrated blue honeysuckle, about 63 Brix, was supplied by H&K Bioscience Co., Ltd. (Seoul, Korea). The brief process for making BHcL was as follows. First, 200 g of the 63 Brix concentrated blue honeysuckle solution was diluted to 25 Brix using distilled water, and then completely lyophilized using a programmable freeze dryer (Operon FDB-5503; Kimpo, Korea). A total of 124.40 g (yield = 62.2%) of BHcL was acquired and stored at  $-20^{\circ}\text{C}$  in a refrigerator to protect the powder from light and humidity until use. Some BHcL specimens were deposited in the herbarium of the Medical Research Center for Globalization of Herbal Formulation, Daegu Haany University (Code BH2013Ku01).

**Proximate analysis of BHcL.** BHcL was dried at  $70^{\circ}\text{C}$  to a constant weight. Moisture content, ash, and crude fiber were determined by the Association of Official Analytical Chemists (AOAC) methods (16). Nitrogen content (N) of the sample was estimated, and the crude protein was calculated as  $\text{N} \times 6.25$ . Total lipids were extracted from the samples with chloroform/methanol (2 : 1, v/v) and gravimetrically quantified. The amount of total carbohydrates was obtained by determining the difference between the weight of the sample taken and the sum of its moisture, ash, total lipid, protein, and fiber content.

### Phytochemical analysis of BHcL.

**Detection of total phenols:** The total phenolic content of the BHcL was determined using the Folin-Ciocalteu colorimetric method (17). The BHcL was oxidized using the Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and the reaction was neutralized with sodium carbonate (Daejung, Siheung, Korea). The absorbance was measured at 760 nm after 120 min at room temperature by an ultraviolet visible (UV-Vis) spectrophotometer (Optizen Pop; Mecasys, Daejeon, Korea) in a 10-mm cell cuvette (NSG Precision Cells, Farmingdale, NY, USA). The absorbance values were then compared with known gallic acid (Sigma-Aldrich, St. Louis, MO, USA) concentration standards. All values were expressed as the mean [mg gallic acid equivalent (GAE)/g of the BHcL sample]  $\pm$  standard

deviation (SD) of three replications.

**Detection of total flavonoids:** The total flavonoid content of the BHcL was determined using a modified colorimetric method (18). Briefly, 0.1 mL of BHcL was mixed with 2.0 mL of deionized water, and subsequently with 0.15 mL of 5% sodium nitrite solution, and was allowed to react for 5 min. Then, 0.75 mL of 2% aluminum chloride (Sigma-Aldrich, St. Louis, MO, USA) was added and allowed to further react for 5 min before 1.0 mL of 1 M NaOH was added. Deionized water was added to bring the final volume of the mixture to 5 mL. The absorbance of the mixture was immediately measured at a 510-nm wavelength against a prepared blank using a UV-Vis spectrophotometer. The flavonoid content was determined by a catechin (Sigma-Aldrich, St. Louis, MO, USA) standard curve and expressed as the mean [mg catechin equivalent (CE)/g of the BHcL sample]  $\pm$  SD of three replications.

**Detection of total anthocyanins:** The monomeric anthocyanin content of the BHcL was measured using a modified pH differential method (19). The BHcL was mixed thoroughly with 0.025 M KCl buffer, pH 1.0. The BHcL was then mixed in a similar manner with a sodium acetate buffer, pH 4.5. UV-Vis spectrophotometry was used to measure the absorbance at 520 and 700 nm against a buffer blank at pH 1.0 and 4.5. Absorbance readings were converted to total mg malvidin-3-O-glucoside (Sigma-Aldrich, St. Louis, MO, USA). The anthocyanin content was calculated as the total monomeric anthocyanin (TMA; mg/g) =  $(\Delta A \times \text{Mw} \times 1,000) / \epsilon \cdot c$ .  $\Delta A = (A_{520 \text{ nm, pH} = 1.0} - A_{700 \text{ nm, pH} = 1.0}) - (A_{520 \text{ nm, pH} = 4.5} - A_{700 \text{ nm, pH} = 4.5})$ , where A is absorbance, Mw (493.5) is the molecular weight of M3G,  $\epsilon$  (28,000, pH 1.0, in methanol solvent) is the molar absorptivity of M3G, and c is the concentration of the grape extract in mg/mL. The anthocyanin content was expressed as mg of malvidin-3-O-glucoside equivalent (M3GE)/g of BHcL for three replications.

**Measurement of betaine by high-performance liquid chromatography (HPLC):** The HPLC system (Agilent 1100 series; Agilent Technologies, Santa Clara, CA, USA) and an Agilent G1315B Diode Array Detector (DAD; Agilent Technologies, Santa Clara, CA, USA) was used for betaine analysis of the BHcL prepared in this study. Empower<sup>TM</sup> Data System software was used to record the output signal of the detector. A YMC-Pack<sup>TM</sup> Polyamine II column (4.6  $\times$  250 mm, 5  $\mu\text{m}$ ; Shimadzu Corp., Tokyo, Japan) was used for separation. The mobile phase was comprised of distilled water and acetonitrile (85:15; Sigma-Aldrich, St. Louis, MO, USA), with the gradient elution system at a flow rate of 1.0 mL/min. The injection volume was 10  $\mu\text{L}$ . The detection UV wavelength was set at 210 nm. The column temperature was set at room temperature. Standard stock solutions of betaine (Sigma-Aldrich, St. Louis, MO, USA) were prepared by dissolving the betaine in 1 mL distilled water to a concentration of 1  $\mu\text{g}/\text{mL}$ . Working stan-

standard solutions were made by diluting the standard stock solution with distilled water. Standard stock solutions and working solutions were stored at 4°C. To prepare the samples, 1 g of lyophilized concentrated BHcL was weighed and dissolved in distilled water at a concentration of 20 mg/mL. Prior to HPLC analysis, the sample preparation was filtered through a 0.45- $\mu$ m filter. Betaine content was expressed as % in BHcL for three independent measurements.

#### **Single dose toxicity test.**

##### **Experimental animals and administration of BHcL:**

Female and male specific pathogen-free ICR mice (n = 20 for each sex, 6 weeks old upon receipt; OrientBio, Sungnam, Korea) were used after a 9-day acclimatization period. Animals were housed (five per polycarbonate cage) in a temperature- (20~25°C) and humidity-controlled (40~45%) room. The light:dark cycle was 12 hr : 12 hr, and food (Samyang, Korea) and water were available *ad libitum*. All animals fasted overnight (~18 hr) before treatment and terminal necropsy. This animal experiment was conducted according to international regulations for the use and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee of Daegu Haany University (Gyeongsan, Korea) [Approval No. DHU2014-071, 2014.10.16]. After the 9-day acclimatization period, the animals were allocated into eight groups based on body weight, with five mice per group (males: 32.38  $\pm$  1.58 g, range 29.1~34.7 g; females: 27.33  $\pm$  1.28 g, range 24.6~30.0 g). The highest dosage used in the present study was 2,000 mg/kg body weight (bw) in a volume of 20 mL, the limited dosage for rodents and the recommended oral dose volume in mouse (15,20), using distilled water as vehicle. For the middle and lower dosage groups, 1,000 and 500 mg/kg bw were selected, respectively, as recommended by KFDA Guidelines (15). In addition, female and male vehicle controls were included in our study. The test article was orally administered once using a sonde attached to a 1-mL syringe. In vehicle control mice, 20 mL/kg bw of distilled water was administered once by gastric gavage, instead of a test solution.

**Clinical signs and body weight:** Clinical signs were recorded based on the Functional Observational Battery (FOB) at least twice a day, before and after dosing (21). Body weights were measured prior to treatment on the day of vehicle or drug administration (Day 0) and on Days 1, 2, 7, 13, and 14 days after administration.

**Necropsy:** All animals that spontaneously died were grossly observed immediately after discovery. All animals that survived the dosing were subjected to terminal necropsy. Animals were asphyxiated by carbon dioxide and gross necropsies were performed in all animals on Day 14 after overnight fasting (about 18 hr, water was not restricted).

**Organ weight measurements and sampling:** The absolute organ weight was measured, and then the relative organ weight (% of body weight) was calculated. The following organs were collected for histopathological observation: lung, heart, thymus, left kidney, left adrenal gland, spleen, left testis or ovary, liver, splenic lobe of the pancreas, brain, left epididymis or total uterus, and left submandibular lymph node. Organs were measured and samples were collected.

**Histopathology:** Samples were fixed in 10% neutral buffered formalin. After 18 hr of fixation, samples were embedded in paraffin and 3- $\mu$ m thick sections were prepared using routine histological methods. Representative sections of each specified organ were stained with hematoxylin-eosin for examination using light microscopy.

**Statistical analyses:** We conducted multiple comparison tests for different dose groups. Variance homogeneity was examined using the Levene test (22). If the Levene test indicated that there were no significant deviations from variance homogeneity, the obtained data were analyzed using a one-way analysis of variance (ANOVA) followed by Scheffé's test to determine which pairs were significantly different in the group comparison. When significant deviations from variance homogeneity were observed following Levene's test, a non-parametric comparison test, the Mann-Whitney U (MW) test, was conducted to determine the specific pairs that were significantly different in the group comparison (23). The 50% lethal dose (LD<sub>50</sub>) and 95% confidence limits were calculated by the Probit method. Statistical analyses were conducted using SPSS for Windows (Release 14.0K; SPSS Inc., Chicago, IL, USA) and a *p*-value of less than 0.05 was considered to indicate a significant difference. According to previous reports (4,5), clinical signs as well as gross and histopathological findings were subdivided into three degrees based on the severity of the changes: 3+, severe; 2+, moderate; and 1+, slight.

## **RESULTS**

**Proximate analysis of BHcL.** The BHcL had an energy value of 3.80 kcal/g and contained 0.93 g/g of carbohydrate, 0.41 g/g of sugar, 0.02 g/g of protein, and 0.20 mg/g of sodium. BHcL did not contain total lipids, saturated lipids, trans fats, or cholesterol (0 mg/g for all) (Table 1).

**BHcL phytochemical profile.** BHcL was comprised of 4.54  $\pm$  0.09% betaine, 210.63  $\pm$  23.65 mg GAE/g total phenols, 159.30  $\pm$  12.51 mg CE/g total flavonoids, and 133.57  $\pm$  4.06 mg M3GE/g total anthocyanins (Fig. 1 and Table 1).

**Mortalities.** No BHcL treatment-related mortalities were observed in male or female mice up to 2,000 mg/kg, the limited dosage in rodents. All animals (5/5; 100%) in all

**Table 1.** Proximate analysis and phytochemical compositions of BHcL

Component	Contents
Proximate analysis	
Energy	3.80 kcal/g
Carbohydrate	0.93 g/g
Sugar	0.41 g/g
Protein	0.02 g/g
Total lipid	0 mg/g
Saturated lipid	0 mg/g
Trans-fat	0 mg/g
Cholesterols	0 mg/g
Sodium	0.20 mg/g
Phytochemical component	
Betaine	4.54 ± 0.09%
Total phenols	210.63 ± 23.65 mg GAE/g
Total flavonoids	159.30 ± 12.51 mg CE/g
Total antocyanins	133.57 ± 4.06 mg M3GE/g

BHcL = Blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae) concentrated and lyophilized powder, test material.

Phytochemical component are expressed as mean ± SD of three independent measurements.

CE = Catechin equivalents.

M3GE = Malvidin-3-O-glucoside equivalents.

GAE = Gallic acid equivalents.

test groups, including both female and male mice, survived during the 14-day experimental period; therefore, all animals were subjected to the terminal necropsy (Table 2).

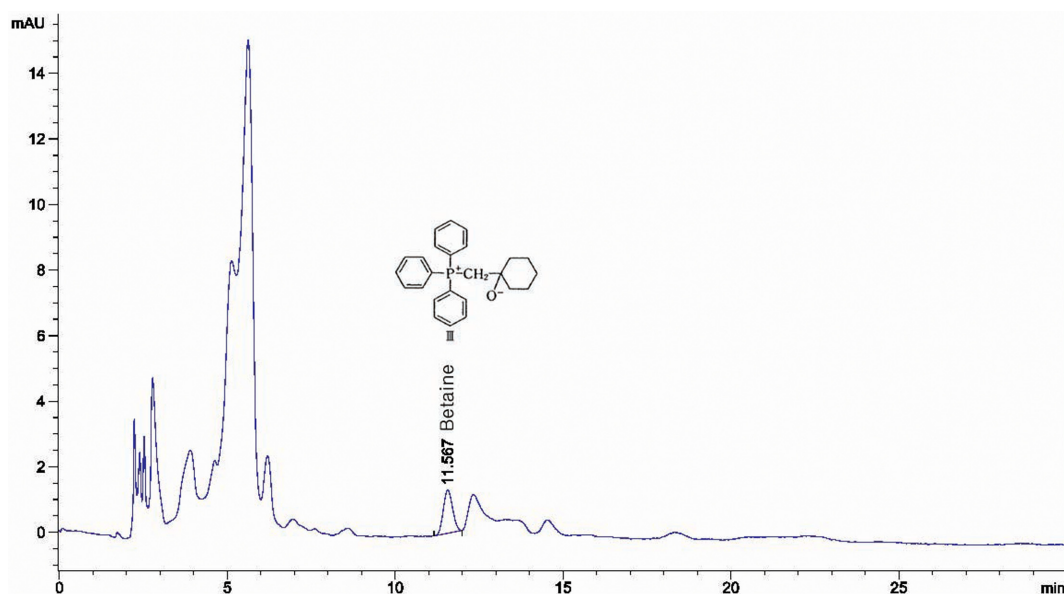
**Clinical signs.** No BHcL treatment-related clinical signs were observed over the 14-day experiment.

**Changes in body weight.** No meaningful body weight changes were detected in any BHcL-treated mice when compared with sex-matched vehicle controls (Fig. 2).

**Changes in organ weight.** No significant organ weight changes were detected in any BHcL-treated mice when compared with sex-matched vehicle controls (Table 3 and 4).

**Necropsy findings.** No BHcL treatment-related gross findings were observed in the present study. We sporadically detected slight [1+] to moderate [2+] lung congestion, splenic atrophy or hypertrophy, edematous uterus changes, and hypertrophy of submandibular lymph nodes in all experimental groups tested, including the sex-matched vehicle controls (Table 2).

**Histopathological findings.** We sporadically detected slight [1+] or moderate [2+] lung congestion spots (i.e., thickening of the alveolar septa with inflammatory cell infiltration and focal hemorrhages), cyst formation in the kidney, decreased splenic white pulp in lymphoid cells, hyperplasia of splenic red pulp in lymphoid cells, uterus mucosa desquamation, and diffused hyperplasia of lymphoid cells in the submandibular lymph nodes in all the experimental groups tested in the present study, including sex-matched vehicle controls. We also observed focal inflammatory cell infiltration in the liver parenchyma with/without focal hepatocyte necrosis in two female BHcL-treated mice: one (1/5; 20%) treated with 1,000 mg/kg and one (1/5; 20%) treated with 500 mg/kg BHcL (Table 2).



**Fig. 1.** Representative high performance liquid chromatogram of betaine in BHcL. Note that BHcL used in this study contains 4.54% of betaine, ranged in 4.45~4.63% at HPLC system based analysis. BHcL, Blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae) concentrated and lyophilized powder, test material; HPLC, High performance liquid chromatography.

**Table 2.** Mortality, clinical signs, gross and histopathological findings of animals exposed with BHcL in the single dose toxicity study

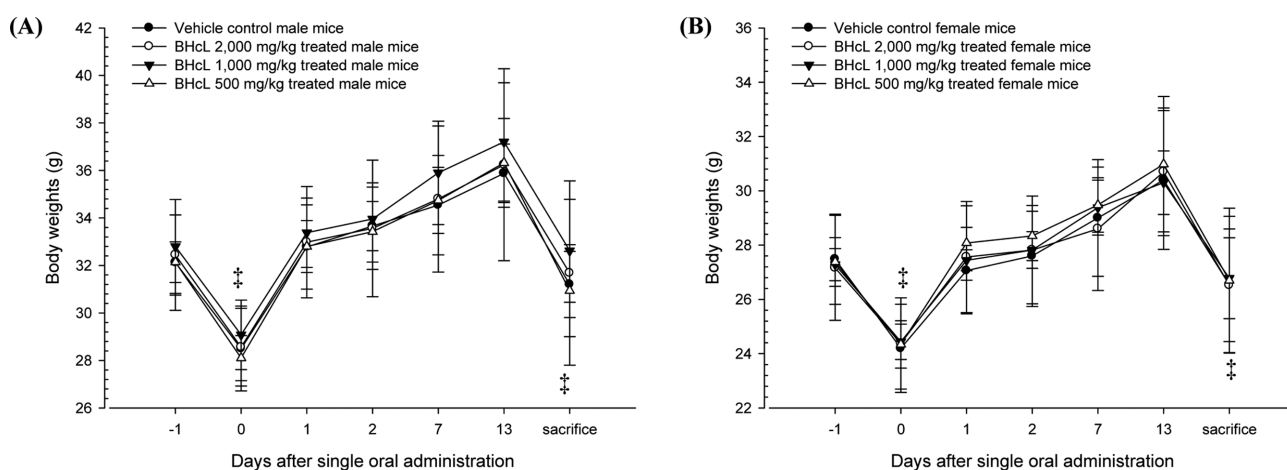
Groups	Male vehicle control	BHcL treated male mice (mg/kg)			Female vehicle control	BHcL treated female mice (mg/kg)		
		2,000	1,000	500		2,000	1,000	500
Mortality	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Clinical signs								
Abnormal signs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Gross findings								
Lung focal congestion 1+	2/5	1/5	1/5	1/5	1/5	0/5	1/5	1/5
Spleen atrophy 1+	1/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5
Spleen hypertrophy 1+	0/5	0/5	0/5	0/5	1/5	1/5	0/5	0/5
Lymph node hypertrophy <sup>a)</sup> 1+	1/5	1/5	0/5	1/5	1/5	1/5	2/5	0/5
Uterus edema 1+					2/5	2/5	1/5	0/5
Uterus edema 2+					0/5	0/5	0/5	1/5
Histopathological findings								
Lung focal congestion 1+	2/5	1/5	1/5	1/5	1/5	0/5	1/5	1/5
Kidney cyst 1+	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen wDE <sup>b)</sup> 1+	1/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5
Spleen rHP <sup>c)</sup> 1+	0/5	0/5	0/5	0/5	1/5	1/5	0/5	0/5
Liver focal inflammation 1+	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5
Lymph node HP <sup>a,d)</sup> 1+	1/5	1/5	0/5	1/5	0/5	0/5	1/5	1/5
Lymph node HP <sup>a,d)</sup> 2+	0/5	0/5	0/5	0/5	1/5	1/5	1/5	0/5
Uterus DM <sup>e)</sup> 1+					2/5	1/5	1/5	1/5

Values were observed animals/total observed animals.

BHcL = Blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae) concentrated and lyophilized powder, test material.

Degrees = 1+, slight; 2+, moderate.

<sup>a)</sup>Submandibular lymph node. <sup>b)</sup>White pulp lymphoid cell decreases. <sup>c)</sup>Red pulp lymphoid cell hyperplasia. <sup>d)</sup>Diffused hyperplasia of lymphoid cells. <sup>e)</sup>Desquamation of the mucosa.



**Fig. 2.** Changes on the body weights during 14 days of observation in female (A) and male (B) mice after single oral treatment of BHcL. No significant changes on body weights were detected in all BHcL treated mice as compared with equal genders of vehicle control mice, respectively. Values are expressed as mean  $\pm$  SD of five mice. BHcL, Blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae) concentrated and lyophilized powder, test material. †, all mice were overnight fasted; before means 1 day before administration. 0 means the day of administration.

## DISCUSSION

In the present study, we investigated the single oral dose toxicity of BHcL in female and male ICR mice to aid in the process of developing natural medicinal ingredients and/or

functional foods. We performed a proximate analysis and measured phytochemical profiles. To investigate toxicity and identify target organs, BHcL was orally administered once to female and male ICR mice at doses of 2,000, 1,000, 500, and 0 mg/kg bw (20 ml/kg bw volume, dissolved in

**Table 3.** Absolute organ weights of animals exposed with BHcL in the single dose toxicity study

Groups	Male vehicle		BHcL treated male mice (mg/kg)		Female vehicle		BHcL treated female mice (mg/kg)	
	control	500	1,000	500	control	1,000	2,000	500
Organ weights (g)								
Lung	0.167 ± 0.009	0.168 ± 0.014	0.173 ± 0.020	0.170 ± 0.014	0.170 ± 0.014	0.175 ± 0.005	0.172 ± 0.010	0.164 ± 0.005
Heart	0.149 ± 0.009	0.140 ± 0.016	0.153 ± 0.009	0.152 ± 0.016	0.132 ± 0.011	0.133 ± 0.005	0.135 ± 0.008	0.126 ± 0.005
Thymus	0.048 ± 0.014	0.040 ± 0.011	0.054 ± 0.014	0.043 ± 0.009	0.065 ± 0.010	0.061 ± 0.016	0.057 ± 0.014	0.056 ± 0.007
Kidney (left)	0.248 ± 0.025	0.269 ± 0.051	0.271 ± 0.036	0.262 ± 0.028	0.167 ± 0.018	0.173 ± 0.016	0.170 ± 0.014	0.167 ± 0.015
Adrenal gland (left)	0.005 ± 0.002	0.004 ± 0.003	0.004 ± 0.003	0.006 ± 0.003	0.007 ± 0.004	0.008 ± 0.003	0.005 ± 0.001	0.004 ± 0.003
Spleen	0.097 ± 0.018	0.087 ± 0.020	0.098 ± 0.014	0.087 ± 0.025	0.111 ± 0.011	0.108 ± 0.015	0.113 ± 0.023	0.113 ± 0.024
Testis/Ovary (left)	0.099 ± 0.011	0.103 ± 0.015	0.103 ± 0.014	0.105 ± 0.004	0.030 ± 0.006	0.035 ± 0.008	0.025 ± 0.008	0.024 ± 0.007
Liver	1.310 ± 0.135	1.308 ± 0.159	1.360 ± 0.048	1.262 ± 0.130	1.100 ± 0.101	1.155 ± 0.111	1.193 ± 0.117	1.154 ± 0.100
Brain	0.471 ± 0.037	0.457 ± 0.026	0.468 ± 0.011	0.483 ± 0.019	0.493 ± 0.014	0.490 ± 0.021	0.500 ± 0.007	0.484 ± 0.031
Lymph node (left) <sup>a)</sup>	0.004 ± 0.003	0.006 ± 0.004	0.006 ± 0.003	0.005 ± 0.002	0.006 ± 0.002	0.007 ± 0.004	0.008 ± 0.004	0.006 ± 0.004
Epididymis (left)	0.042 ± 0.005	0.044 ± 0.004	0.042 ± 0.005	0.041 ± 0.003				
Pancreas (splenic lobe)	0.164 ± 0.020	0.161 ± 0.019	0.166 ± 0.028	0.157 ± 0.042	0.151 ± 0.027	0.163 ± 0.021	0.148 ± 0.021	0.138 ± 0.014
Uterus					0.178 ± 0.051	0.152 ± 0.052	0.145 ± 0.027	0.133 ± 0.042

Values are expressed as mean ± S.D. of five mice.

BHcL = Blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae) concentrated and lyophilized powder, test material.

<sup>a)</sup>Submandibular lymph node.

**Table 4.** Relative organ weights of animals exposed with BHcL in the single dose toxicity study

Groups	Male vehicle		BHcL treated male mice (mg/kg)		Female vehicle		BHcL treated female mice (mg/kg)	
	control	500	1,000	500	control	1,000	2,000	500
Organ weights (% of body weight)								
Lung	0.536 ± 0.026	0.532 ± 0.032	0.532 ± 0.063	0.551 ± 0.008	0.646 ± 0.072	0.656 ± 0.035	0.652 ± 0.074	0.616 ± 0.045
Heart	0.478 ± 0.039	0.475 ± 0.043	0.470 ± 0.043	0.491 ± 0.044	0.501 ± 0.059	0.498 ± 0.015	0.512 ± 0.053	0.476 ± 0.042
Thymus	0.152 ± 0.040	0.125 ± 0.019	0.165 ± 0.041	0.138 ± 0.028	0.244 ± 0.027	0.228 ± 0.055	0.216 ± 0.047	0.217 ± 0.022
Kidney (left)	0.795 ± 0.052	0.849 ± 0.135	0.832 ± 0.116	0.848 ± 0.078	0.633 ± 0.076	0.645 ± 0.052	0.644 ± 0.067	0.627 ± 0.060
Adrenal gland (left)	0.015 ± 0.006	0.013 ± 0.008	0.011 ± 0.008	0.018 ± 0.010	0.026 ± 0.016	0.029 ± 0.012	0.020 ± 0.005	0.014 ± 0.009
Spleen	0.310 ± 0.050	0.272 ± 0.036	0.300 ± 0.039	0.282 ± 0.075	0.417 ± 0.031	0.403 ± 0.056	0.429 ± 0.091	0.419 ± 0.054
Testis/Ovary (left)	0.319 ± 0.035	0.328 ± 0.056	0.319 ± 0.052	0.340 ± 0.026	0.112 ± 0.021	0.129 ± 0.025	0.094 ± 0.030	0.089 ± 0.018
Liver	4.194 ± 0.294	4.129 ± 0.167	4.177 ± 0.148	4.078 ± 0.308	4.161 ± 0.405	4.310 ± 0.314	4.503 ± 0.348	4.333 ± 0.265
Brain	1.509 ± 0.102	1.455 ± 0.135	1.440 ± 0.118	1.567 ± 0.124	1.873 ± 0.197	1.835 ± 0.126	1.895 ± 0.144	1.833 ± 0.263
Lymph node (left) <sup>a)</sup>	0.014 ± 0.009	0.018 ± 0.011	0.018 ± 0.008	0.018 ± 0.007	0.023 ± 0.006	0.026 ± 0.016	0.030 ± 0.012	0.020 ± 0.012
Epididymis (left)	0.136 ± 0.013	0.139 ± 0.021	0.130 ± 0.021	0.134 ± 0.012				
Pancreas (splenic lobe)	0.526 ± 0.053	0.514 ± 0.092	0.509 ± 0.068	0.508 ± 0.130	0.572 ± 0.107	0.609 ± 0.070	0.563 ± 0.104	0.518 ± 0.047
Uterus					0.670 ± 0.188	0.574 ± 0.214	0.545 ± 0.092	0.511 ± 0.203

Values are expressed as mean ± S.D. of five mice.

BHcL = Blue honeysuckle (*Lonicera caerulea* L., Caprifoliaceae) concentrated and lyophilized powder, test material.

<sup>a)</sup>Submandibular lymph node.

distilled water). We then determined the treatment-induced mortality and body weight changes. Further, clinical signs were monitored for 14 days after treatment via gross observation, in addition to examining organ weight changes and principle organ histopathology as compared with sex-matched vehicle control mice, based on the recommendations set forth by the KFDA Guidelines (15).

The KFDA Guidelines (15) recommend that the highest dose of test materials administered is 2,000 mg/kg or the maximum solubility; the guidelines also recommend that the dosage volume is kept below 20 mL/kg, in case of acute toxicity in mice (19). Because there are no available toxicological data following oral BHcL treatment in female and male mice, the highest dosage was selected to be 2,000 mg/kg in a volume of 20 mL, the limited dosage for rodents and the recommended oral dose volume in mice (15,20). In the present study, 1,000 and 500 mg/kg bw were chosen for administration in middle- and low-dose groups, as recommended by the KFDA Guidelines (15). Both female and male vehicle control groups were also included in this experiment. The test material was administered once by gastric gavage using distilled water as a vehicle.

The proximate analysis showed that the BHcL used in this experiment had an energy value of 3.80 kcal/g and contained 0.93 g/g of carbohydrate, 0.41 g/g of sugar, 0.02 g/g of protein, and 0.20 g/g of sodium. BHcL did not contain any total lipids, saturated lipids, trans fats, or cholesterol (0 mg/g for all). BHcL contained  $4.54 \pm 0.09\%$  of betaine,  $210.63 \pm 23.65$  mg GAE/g of total phenols,  $159.30 \pm 12.51$  mg CE/g of total flavonoids, and  $133.57 \pm 4.06$  mg M3GE/g of total anthocyanins.

In chemistry, a betaine is any neutral chemical compound with a positively charged cationic functional group, such as a quaternary ammonium or phosphonium cation. In general, onium ions bear no hydrogen atoms and have a negatively charged functional group, such as a carboxylate group, which may not be adjacent to the cationic site. In biological systems, many naturally occurring betaines serve as organic osmolytes, which are substances synthesized or taken up from the environment by cells for protection against osmotic stress, drought, high salinity, or high temperature. Intracellular accumulation of betaines, which do not perturb enzyme function, protein structure, or membrane integrity, permits water retention in cells, thus protecting from the effects of dehydration (24). Betaines have been used as medicine as well; they appear to protect organs via antioxidative effects (25-27). Because the BHcL used in this study contained 4.54% betaine (range 4.45~4.63% as determined by an HPLC system-based analysis), betaine may be one of the active ingredients found in BHcL, or as standardization ingredients BH extract contains ascorbic acid and phenolic components, particularly anthocyanins, flavonoids, and low molecular weight phenolic acids (6,7); however, this experiment is the first to report

the presence of betaine in BH. In the present study, BHcL also contained  $210.63 \pm 23.65$  mg GAE/g of total phenols,  $159.30 \pm 12.51$  mg CE/g of total flavonoids, and  $133.57 \pm 4.06$  mg M3GE/g of total anthocyanins.

No treatment-related mortalities were observed in either sex within 14 days following a single oral BHcL treatment up to 2,000 mg/kg bw, the limited dosage in rodents. No BHcL treatment-related changes were detected in body or organ weight, clinical signs, necropsy, or histopathological findings as compared with sex-matched vehicle controls.

Regardless of treatment, all mice used in this study had normal body weight and organ weight changes similar to the ranges observed in normal age-matched mice (28). Gross findings included slight or moderate lung congestion, splenic atrophy or hypertrophy, edematous changes of the uterus, or hypertrophy of the submandibular lymph nodes. Histopathological findings included lung congestion spots (i.e., thickening of the alveolar septa with inflammatory cell infiltration and focal hemorrhages), cyst formation in the kidney, decreased splenic white pulp lymphoid cells, hyperplasia of the splenic red pulp lymphoid cells, uterus mucosa desquamation, and diffused hyperplasia of lymphoid cells in the submandibular lymph nodes. These were considered to be accidental findings and not toxicological signs related to BHcL treatment, as they were sporadically detected in all experimental groups tested in the present study, including sex-matched vehicle controls. In particular, the edematous changes and desquamation of mucosa in the uterus were considered to be secondary changes from different physiological estrus cycles (29,30). Additionally, most of these findings were also observed in normal mice as sporadic accidental findings (4,5). Focal inflammatory cell infiltration in the liver parenchyma was observed in two female BHcL-treated mice: one (1/5; 20%) 1,000 and one (1/5; 20%) 500 mg/kg BHcL-treated mouse. Because this finding was not dose-dependent, it was likely not a BHcL treatment-related toxicological effect. Further, it was not detected in female or male mice treated with the highest dosage (2000 mg/kg) of BHcL.

Together, these results suggest that BHcL is not toxic. The LD<sub>50</sub> and approximate LD of BHcL in both female and male mice after single oral treatment were considered to be greater than 2,000 mg/kg bw, as no mortalities were detected up to 2,000 mg/kg bw, the limited highest dosage recommended by KFDA Guidelines (15). Further, no specific organs were found to be abnormal or side effects were observed.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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