Bio-Medical Materials and Engineering 24 (2014) 2025–2039
DOI 10.3233/BME-141012
IOS Press

Effect of balanced low pressure drying of *curcuma longa* leaf on skin immune activation activities

Wooseok Choi[a], Hye Won Lim[b] and Hyeon Yong Lee[c,*]
[a]*Department of Medical Biomaterials Engineering, Kangwon National University, Chuncheon 200-701, Republic of Korea*
[b]*Shebah Biotech Co., Chuncheon Bioindustry Foundation Hi-Tech Venture Town, Chuncheon 200-161, Korea*
[c]*Department of Food Science and Engineering, Seowon University, Cheongju 361-742, Republic of Korea*

Abstract. The effect of balanced low pressure drying pretreatment associated with ultrasonication extraction (BU) on the enhancement of skin immune modulatory activities of *Curcuma longa* leaf was studied by comparing with conventional hot air drying (HE), freeze drying (FE) and balanced low pressure drying (BE) pretreatment processes. In considering skin immune activation activities such as the inhibition of hyaluronidase activity, the BU extract showed ca. 10% higher than those of HE, and even higher than that of the FE extract. Nitric oxide production from macrophage of the BU extract in adding 1.0 mg/mL was increased up to 16.5 μM. When measuring inhibition of IL-6 and TNF-α production from the human T lymphocytes (T cell), the BU extract also showed 53% and 78% of inhibition effect, respectively. It is found that the BU extract could effectively suppress the expression levels of skin inflammation related genes such as Cox-2 and iNOS, down to 80% and 85% compared to the control, respectively. Balanced low pressure drying process was especially active on dehydration of the leaves with minimizing the destruction and making easier elution of the bioactive substances, which resulted in higher extraction yield and better biological activities.

Keywords: Curcuma longa, skin immune activation activities, balanced drying process, ultrasonication extraction process

1. Introduction

Turmeric (*Curcuma longa*) is a perennial plant of the ginger family, Zingiberaceae, and genus Curcuma [1,2]. Flower resembling small, thorn-like fine hair grows behind the leaves. *C. longa* originated from tropical Asia, and it grows in tropical and southern regions of China [1]. In galenic pharmacy where the effects of oriental medicines are listed, *C. longa* is known to have an excellent pain relief effect and enhances the blood circulation due to its warm characteristics. *C. longa* is traditionally used for treating inflammations in several countries [2]. Also, *C. longa* is known to have coloring effect due

*Corresponding author: Hyeon Yong Lee, Department of Food Science and Engineering, Seowon University, Cheongju 361-742, Republic of Korea. Tel.: +82-43-299-8471; Fax: +82-43-299-8471; E-mail: hyeonl@seowon.ac.kr.

to curcuminoids and recently its bioactive and medicinal effect is recognized. This leads to intense studies carried out on strengthening of an immune system, hepatitis, cholangitis, cholelithiasis, catarrhal jaundice, effects in digestive system, cardiovascular system, antiplatelet aggregation, a blood lipid drop, antioxidation, anti-mutation, anti-tumor, and antibiosis [1, 3–5].

So far, the study has been focused on curcuminoids contained in *C. longa* on screening the biological activities of antioxidation [4], anti-cancer [1], anti-inflammation [5,6], and anti-virus [7] while there are no studies on actual enhancement of the activities and processes for enhancing curcumin contents that are the main active ingredients in *C. longa*. Also, the focus of existing studies is limited to the stems and roots of *C. longa*. leaves and flowers of *C. longa* that are known as byproducts are rarely used, and there are almost no studies are conducted on the destruction of the ingredient and alteration of its characteristics according to extra processes such as extraction compared to the roots and stems [8].

However, only the root of *C. longa* has been used. For the large quantity leaves, which are the typical byproducts of *C. longa,* only small part of them are used as feed stock while the rest are disposed which causes environmental problems [9].If the byproducts and waste resources of leaves and flowers of *C. longa* can be used to make advanced materials, more added value could be generated. Especially for the leaves that have large volume and surface area, low temperature processes with short time are necessary since they are vulnerable to alteration followed by severe physical stress and high heat processing. The leaves of C. *longa* have very weak tissue connected to the roots, and prone to the large loss of active ingredients and degeneration of the material. Thus, high temperature drying as a pretreatment is inevitable before using the leaves as various materials [10].

In general, in drying the medicinal plants, the hot air drying is the most typical and conventional process among other drying pretreatment processes. However, long drying time and high temperature could damage the active ingredients exist in thin and soft surface and inner side of the *C. longa* leaf and degrade the product quality [11]. High temperature is the biggest problem in hot air drying method [12]. Therefore, a balanced low pressure drying method was introduced to reduce the loss of active ingredients even though in general a freeze drying would be useful; however, a freeze drying process is considered to be very expensive and may not be feasible for large industrial scale productions. To overcome this limitation in drying relatively soft plant materials, the balanced low pressure pretreatment process could be employed since it has as high yield as freeze drying process even though it requires relatively large plant equipment and systems such as large size air blowing system and holding device for maintaining regular pressure inside the chamber [10,13]. Through this drying process, the alteration of the material and the damage are minimized since the leaves are dried under balanced drying conditions with constantly reducing the humidity at low pressure and low temperature. This process is a relatively new method to dry thin and soft plant materials even though this drying process could save time and cost, and also avoid the desiccation of thin parts of the leaves and the alteration of active ingredients by high temperature. Thus, in this work, balanced low pressure drying was selected as a treatment process that can yield the same high drying efficiency as freeze drying method with relatively low price [13].

In order to reduce the desiccation of thin leaves, loss of thin surfaces and active ingredients, a balanced low pressure drying will be associated with ultrasonication extraction process since ultrasonication extraction process is known to be useful for extracting thin leaves with fragile surfaces while it generates substantial energy by inducing the cavitation in the fluid due to ultrasonic vibration [14]. Also, ultrasonic energy induces high pressure by the impact effect and at the same time the kinetic energy of reaction molecules increases. Therefore, it will be appropriate for extracting the leaves that should be processed within the least amount of the time. This process could expect a high yield and

reduced extraction time than the conventional hot water extraction process [15, 16]. Therefore, this study is conducted to increase the extraction yield of *C. longa* leaf, which was considered as less valuable byproduct than stems and roots, through complex processes including balanced low pressure pretreatment followed by ultrasonication extraction. Furthermore, by identifying the enhanced curcumin contents, which is an excellent ingredient for immune activity and anti-inflammation, various methods has been used to identify the immune activities contained in *C. longa* leaves through complex process as a cosmetic material.

2. Materials and methods

2.1. Cell cultures and sample preparation

Cell lines used for the experiment were human skin fibroblast (CCD-986sk, KCLB 21947, Korea), mouse melanoma (Clone M-3, KCLB 10053.1, Korea), and mouse macrophage (RAW 264.7, KCLB 40071, Korea). Dulbeco's Modified Eagle's Medium (DMEM) (GIBCO, USA) and RPMI 1640 (GIBCO, USA) media enriched with 10% fetal bovine serum (GIBCO, USA) were used. Hepes buffer (SIGMA, USA), gentamycin sulfate (SIGMA, USA), bovine serum albumin (SIGMA, USA) and trysin-EDTA (SIGMA, USA) were also used as reagents for culturing the cells.

Leaves of *C. longa* were collected in Jeollabuk-do, Korea, September, 2011 and dried by three different methods: hot air drying, freeze drying extraction, and balanced low pressure drying. For hot air drying, collected leaves were dried by using a hot air dryer (CF-21WF, Jeiotech, Korea) at 85°C with hot air circulation velocity of 5 m/h for 72 hours. For freeze drying, a freeze dryer (PVTFA 10AT, Ilshin Autoclave, Korea) was used under 10 mm Torr at -70°C for 72 hours. For balanced low pressure drying, a tray type low pressure drying device (VS-1202V5, Vision Scientific, Korea) was used under 780 hPa at 35°C with 10 m/h of cold dehumidified air flow rate for 72 hours [10]. 100 g of dried leaves from each different drying process was extracted with 1L of 70% ethyl alcohol by a thermal extractor (TL200, Misung Scientific Co., Korea) at 80°C for 24 hours. For ultrasonication extraction (BU), 100 g of dried leaves from a low pressure dryer was extracted by an ultrasonic extractor (AUG-R-900, Asia Ultrasonic, Korea) at 120 kHz frequency and 25°C for 3 hours, then again extracted by a thermal extractor (TL200-6Point(K), Misung Scientific Co., Korea) with 70% ethanol at 80°C for 12 hours. Each extract was filtered through 20-25 μm filter paper (NO.541 Filter Paper, Whatman, USA) and concentrated by a rotary vacuum evaporator (N-N series, Eyela, Germany), and then freeze dried to make the powder. It was stored at -4°C before use.

2.2. Measurement of curcumin in the extracts from different drying processes

The amounts of curcumin in the extracts from each drying process were estimated by a High Performance Liquid Chromatography (HPLC) (500 series, BIO-TEK instrument, Italy). As a standard, 250 ppm of curcumin (08511, Sigma, USA) was dissolved in distilled water for HPLC analysis, and then filtered by a 0.2 μm syringe filter. 2000 ppm of each sample was also dissolved in distilled water and filtered with a 2 μm syringe filter. HPLC was equipped with a BIO-TEK 522 controller pump and BIO-TEK HPLC 535 UV Detector (254 nm). For the columns, CAPCELL (C-18) packed column (4.6 mm × 250 mm, 5 μm) was used. For mobile phase, methanol was allowed to flow with the velocity of 1.2 mL/min at 25°C. Injection volume was 25 μL and it was injected into the mobile phase [17].

2.3. Measurement of cell toxicity

Cell toxicity was measured by Mosmann method using 3-(4,5-Dimethythiazol-2-yl)-2,5-Diphenyl-tetrazoliumbromide (MTT) as follows: 1.5×10^4 viable cells/well of CCD-986sk cells were inoculated into a 96-well plate, and when the cells reach approximately 80% confluency, the extracts from each process were added into the each well with the final concentration of 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL. Cells are then cultured in a CO_2 incubator (CB150, Binder, Germany) for 24 hours. MTT solution in a concentration of 5 μg/mL was added to each well afterwards, and it was allowed to rest for four hours. After four hours, supernatant was removed and 10 μL of acid-isopropanol (0.04N HCl in isopropanol) was added. Absorbance was then measured at the wavelength of 565 nm using a 96 well microplate reader (EMax Endpoint, Sunnyvale, USA). Cell toxicity was calculated as a percentage of formazan crystal formed by MTT reagent to its control group [18].

2.4. Measurement of hyaluronidase inhibitory activities

Reissig assay was used for the measurement of hyaluronidase inhibitory activities as follows: 7900 unit/mL of hyaluronidase was dissolved in 0.1 M acetate buffer (pH 3.5). In 50 μL of this solution, *C. longa* extracts samples were added by 20 μL to make final concentration of 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL. Dimethyl sulfoxide (DMSO) solution was added to the control group. 200 μL of $CaCl_2$ (12.5 mM) was mixed and it was stored in 37°C water bath for 20 minutes for the enzyme activation. Hyaluronic acid (12 mg/5mL) was then dissolved in 0.1 M acetate buffer (pH 3.5), and 250 μL of this solution was added to activated hyaluronidase solution. This mixture was stored in 37°C water bath for 40 minutes. After culturing, 100 μL of 0.4 NaOH solution and 100 μL of 0.4 M potassium tetraborate were added to the mixture. Then, put into a boiling water bath for 3 minutes and cooled rapidly. 3.28 mL of dimethyl aminobenzaldegye solution (mixed solution of p-dimethyl amino-benzaldehyde 4 g, 100% acetic acid 350 mL, 10 HCl 50 mL) was added to cooled mixture and it was cultured in 37°C water bath for 20 minutes before its absorbance was measured at 585 nm. Hyaluronidase inhibitiory activities are calculated by the below Eq. (1) [19].

$$Hyaluronidase\ \ Inhibition\ \ activity(\%) = \frac{control\ \ O.D. - sample\ \ O.D.}{control\ \ O.D.} \times 100 \quad (1)$$

2.5. Measurement of Nitric Oxide (NO) production

The secretion of Nitric Oxide (NO) from macrophage cell RAW264.7 was measured after the addition of the *C. longa* extracts. The nitrite present in the culture medium was determined using a modified Griess reaction [20]: The cells were seeded into 96-well plates at a concentration of 1.0×10^4 cells/mL. Afterwards, Lipopolysaccharides (LPS, L6529, Sigma, USA) 100 μg/mL was added to this mixture, which was then cultured in a 37°C, 5% CO_2 incubator for 24 hours. Curcumin was used as a standard, and 50 μL of Griess reagent and 50 μL of cell culture supernatant were mixed and reacted for 5 min in a 96-well plate. The amounts of NO production were measured by a 96 well ELISA Reader (EMax, Sunnyvale, CA, USA) with $NaNO_2$ utilized as a standard at 540 nm.

2.6. Measurement of IL-6 and TNF-α secretion from human T cell

The secretion of IL-6 and TNF-α from human T lymphocytes (T cell) were measured with the Enzyme-Linked ImmunoSorbent (ELISA) assay kits (Biosciences/Pharmingen, Chemicon, USA) by the following procedure: 5.0×10^4 viable cells/mL of human T lymphocytes (T cell) was inoculated into a 6-well plate, then the cells were cultured at 37°C in a 5% CO_2 incubator for 24 hours before being treated with 100 μL of the extracts in different concentrations. After one hour treatment, the cells were added with 1 μg/mL of Lipopolysaccharides (LPS) (L6529, Sigma, USA) to induce the inflammation. After 12 hours, the supernatant was collected from the wells, and the absorbance was measured using an ELISA reader (EMax Microplate, Sunnyvale, USA) at 450 nm, and the amounts of each IL-6 and TNF-α were also measured and compared with the control group (not treated cells) by the 96 well ELISA reader at 450 nm [21].

2.7. Measurement of the expression levels of Cox-2 and iNOS

5.0×10^4 viable cells/well of RAW 264.7 cells grown in low glucose free-serum DMEM with BSA were inoculated into a 6-well plate at 37°C in a 5% CO_2 incubator for 24 hours. Then, α-MSH was added for one hour until it reached 10 nM, and the extracts from each process were added in different concentrations. After 24 hours cultivation, 1 μg/mL of LPS was then treated for 12 hours. After 12 hours, the cells were washed with PBS before Trizol (15596-026, Invitrogen, USA) was added. With the scrapper, cells were harvested and the harvested cells were put into a microfuge tube. It was the stored in room temperature (15-30°C) for 10 minutes. Afterwards, total RNA was separated using chloroform (650498, Sigma Aldrich, USA) and isopropanol (W292907, Sigma Aldrich, USA). Total RNA was washed with ethanol, air-dried for 5-10 minutes, and the pellet was mixed with RNA free water (10977-023, Invitrogen, USA). The mixture was incubated at 55°C for 5 minutes and then stored at -70°C. Total RNA obtained was diluted by 100-fold and the absorbance was measured at 260 nm and 280 nm using Nanovue spectrophotometer (28-9569-66, GE Healthcare, USA) in order to identify the purity and the quantity of the RNA. cDNA synthesis using total RNA was conducted using Super-Script III First-Strand Synthesis System (18080-051, Invitrogen, USA). For Real-time PCR (iQ5 & MyiQ Cycler, BioRad, USA), cDNA was diluted by 25-fold and the DNA was amplified using iNOS primer (forward, reverse) and Cox-2 primer (forward, reverse), DEPC-water, SYBR Green Supermix (170-8880, Bio-rad, USA) in order to identify the expression of iNOS (forward -GTTCTCAAGGCACAGGTCTC-, reverse -GCAGGTCACTTATGTCACTTAT-, Invitrogen, USA) and Cox-2 (forward -CAAAAGCTGGGAAGCCTTCT-, reverse -CCATCCTTGAAAAGGCGCAG-, Invitrogen, USA). Specificity of the PCR products of iNOS, COX-2 and GAPDH were confirmed by melting curve analysis and agarose gel electrophoresis. All real time PCR analyses were performed in triplicate [22].

2.8. Statistical analysis

The experimental data were analyzed using the statistical software SAS (Statistical Analysis System) and the analyzed values were presented as the mean ± S.D. The significance of differences were analyzed by ANOVA (analysis of variance) and DMRT (Duncan's multiple range tests) at the level of $\alpha = 0.05$.

3. Results and discussion

3.1. Comparison of extraction yields and curcumin contents from different drying processes

The balanced low pressure drying and ultrasonication extraction (UE) method showed the highest yield of 13.20%, followed by 12.90% of a freeze drying by 70% ethanol extraction (FE), 12.11% of balanced drying by only ethanol extraction without UE and 11.78% of hot air drying (HE) as shown in table 1. It was interesting that the extraction yield from BE was not much different from that from FE that was relatively expensive and time consuming process, possibly because of less shrinkage of the leaf structures during balanced low temperature drying under low pressure condition [10,13]. Related to hard structured materials such as stems and roots, the leaves become desiccated and crumbled when they went through hot air pretreatment before going through the main process, the hot water extraction. This is considered as a reason for yielding of low powder extraction. Hence the balanced low pressure dying would be an efficient process for hydrating weak structures. After applying ultrasonication process, the extraction yield was greatly increased up to 13.20%, which is much higher than that from freeze drying process. The extraction yield will also be increased if the extract from the freeze drying process is applied to ultrasonication extraction. However, it would not be feasible for industrial applications since it would require much labor and processing time since a freeze drying process itself is time and labor consuming as well as less economic, compared to simple balanced drying process.

In general, the pretreatment process should be selected by considering the characteristic of the specimen before proceeding to the main process when the extraction process was being carried out. It is considered that balanced low pressure extraction pretreatment process and ultrasonication extraction process is more efficient than freeze drying process with a high cost when extracting the leaves due to easy diffusion of active ingredients and shorter travel distance between extract and solvent by breakdown of cell structure caused by inner pressure difference inside the cell and high pressure by cavitation around the *C. longa* leaves with balanced low pressure drying pretreatment process and ultrasonication extraction process at 120 kHz [16]. Also, balanced low pressure drying pretreated *C. longa* leaves maintained its inner tissue bond during the process and it enabled the extraction of the ingredients with ultrasonication methods that were difficult to be done with existing extraction methods [23]. Hard and firm tissues such as roots and stems were affected mainly by the amount of physical energy from the outside such as ultrasonic waves. However, soft tissues such as *C. longa* leaves used in this study yield high difference in extraction yield, breakdown of leaf structure and substance according to pretreatment drying method that maintains the extract in its optimal condition than the influence of outside energy such as ultrasonication.

The amounts of curcumin, a key bioactive substance in *C. longa*, extracted from different drying and extraction conditions from the extract of BU summarized in Table 2 after being analyzed by HPLC. The highest amounts of curcumin were estimated as 71.840 μg/mg in the extract from the BU and followed by 70.256 μg/mg, 68.912 μg/mg and 66.785 μg/mg in the FE, the BE and the HE, respectively. The highest concentration of 71.840 μg/mg in the BU was about 70% of the amounts existed in the roots that are known to be most existed in *C. longa*. This result also indicates that the new drying method can increase the elution of curcumin and result in enhancing skin anti-inflammation activities, which should expand the application of the byproducts of *C. longa* with a less expensive pretreatment process.

Table 1

Extraction yield of *Curcuma longa* leaf from different drying and extraction conditions

Sample*	Solvent	Time (h)	Temp. (°C)	Ultrasonication (kHz)	Yield (%)***
HE	70% ethyl alcohol	24	80	-	11.78 ± 0.31^{A}
FE					12.90 ± 0.41^{B}
BE					12.11 ± 0.24^{C}
BU**		12		120	13.20 ± 0.15^{D}

Note: HE: Hot air drying extraction; FE: Freeze drying extraction; BE: Balanced low pressure drying extraction; BU: Balanced low pressure drying and ultrasonication extraction.

** The extract from ultrasonication extraction at 120 kHz and 25°C for 3 hours after being dried by Balanced low pressure drying process (BU).

*** Mean values±SD from triplicate separated experiments are shown. Means with difference letter (A-D) within a sample are significantly different at $p<0.05$.

Table 2

Comparison of curcumin contents of the extracts from different drying methods

Sample*	Curcumin content (μg/mL) **
HE	66.785 ± 0.28^{A}
FE	70.256 ± 0.16^{B}
BE	68.912 ± 0.16^{C}
BU	71.840 ± 0.23^{D}

Note: *HE: Hot air drying extraction 2000 ppm; FE: Freeze drying extraction 2000 ppm; BE: Balanced low pressure drying extraction 2000 ppm; BU: Balanced low pressure drying and ultrasonication extraction 2000 ppm.

**Mean values ± SD from triplicate separated experiments are shown. Means with difference letter (A-D) within sample are significantly different at $p<0.05$.

3.2. Measurement of cytotoxicity of the extracts from different drying processes

Figure 1 demonstrates that the cell toxicity of the extracts increased as the treatment concentration increased, and the highest cytotoxicity was observed in adding the extract from hot drying process (HE) as 12.84% at 1.0 mg/mL with highest addition. The extracts from FE and BU showed the lowest cell toxicity at all concentrations without much difference among FE, BU and BE in adding the same concentrations. This result suggests that the potential toxic residues could be generated from relatively weak leaves during high temperature drying, which would not be observed for the case of drying the roots and stems, etc. Moreover, the extracts from the *C. longa* leaves have relatively low cell toxicity, compared to 20.12% of *Acer mono* extract at 1 mg/mL concentration [24]. These results could also suggests that the active ingredients in *C. longa* leaves were not lost by balanced low pressure drying pretreatment but maintained its shape while being extracted by cavitation phenomenon induced by ultrasonication extraction process under low temperature condition [25]. When balanced low pressure drying pretreatment process and ultrasonication extraction process is introduced to natural substances that are composed of soft tissues such as *C. longa* leaves, bioactive substances and highly functional active substances could be efficiently obtained. The natural substances that are also more improved with high contents related to existing process. Therefore, the BU extract with the least cell toxicity against human fibroblast cell can be considered as possible cosmetic material that has the least irritation on the skin.

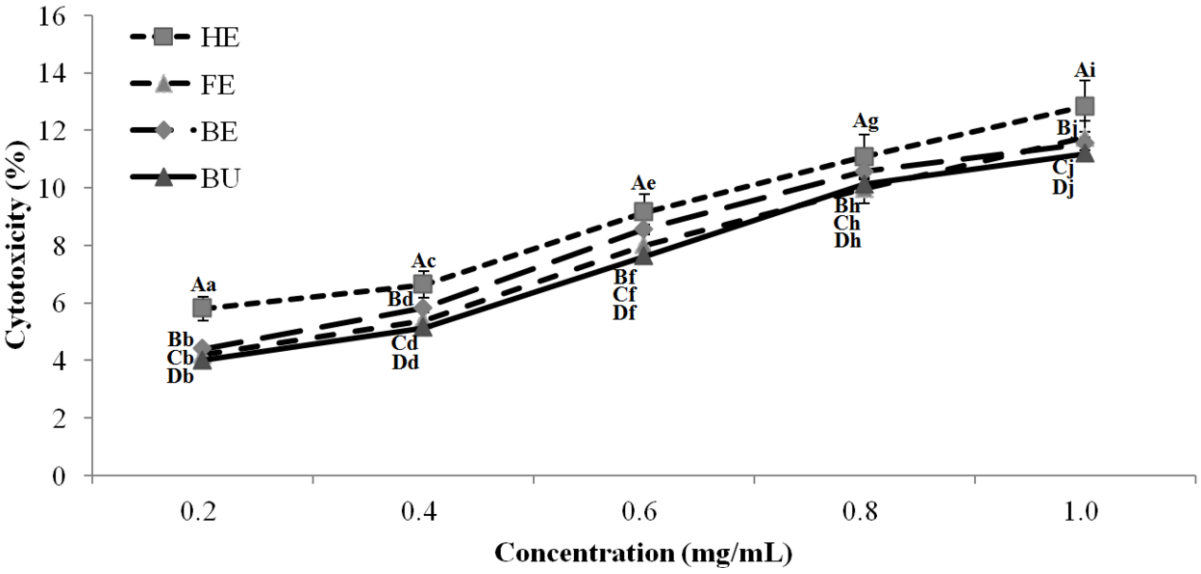

Fig. 1. Cytotoxicity of the extracts of the *Curcuma longa* leaf from several different drying methods.
*HE: Hot air drying extraction; FE: Freeze drying extraction; BE: Balanced low pressure drying extraction; BU: Balanced low pressure drying and ultrasonication extraction.
**Mean values±SD from triplicate separated experiments are shown. Means with difference letter (A-D) within same sample are significantly different at $p<0.05$ and mean with difference letter (a-j) within a same concentration are significantly different at $p<0.05$.

3.3. Measurement of skin immune activation activities of the extracts

3.3.1. The inhibition of hyaluronidase activities

The use of skin immune activation activity has often been observed by the degree of inhibition hyaluronidase enzyme since hyaluronidase can break down the hyaluronic acid that helps the moisturization as well as firmness of the skin, and it is known as skin immunity inhibitory factor [26]. As shown in Figure 2, the inhibition of hyaluronidase activities gradually increased as the addition of the samples increased by the order of BU, FE, BE and HE for all the ranges of the concentrations even though there was not much different between the extracts from BU and FE in general. This trend was very similar to those of extraction yield and cytotoxicity in Table 1 and Figure 1, which also strongly indicates that the extracts from FE and BU could contain more bioactive substances with less toxic residues than that of HE and BE. The BU showed the highest hyaluronidase inhibitory activities of 43.12%. Also, the extracts from BU generally showed slightly high hyaluronidase inhibitory activities than that of FE all concentrations. Compared to the HE, the BU also showed maximum 9% hyaluronidase inhibitory activities at 1.0 mg/mL. Moreover, the extract from BU showed ca. 30% lower hyaluronidase inhibitory activities compared to a positive control, Ibuprofen which is known as a great immune stimulator, and whose activity was better than those from other medicinal plants: Compared to 46% and 35% hyaluronidase inhibitory activities from the ginseng extract and *Paeonia japonica* extract at the concentration of 10 mg/mL, respectively, these results indicate that *C. longa* leaf extract have high hyaluronidase inhibitory activities even at low concentration as well as enhanced efficiency for transformation, formation and maintenance to hyaluronic acid [27]. Thus, synergy of both physiological immune activities within the body and anti-inflammation effect were expected.

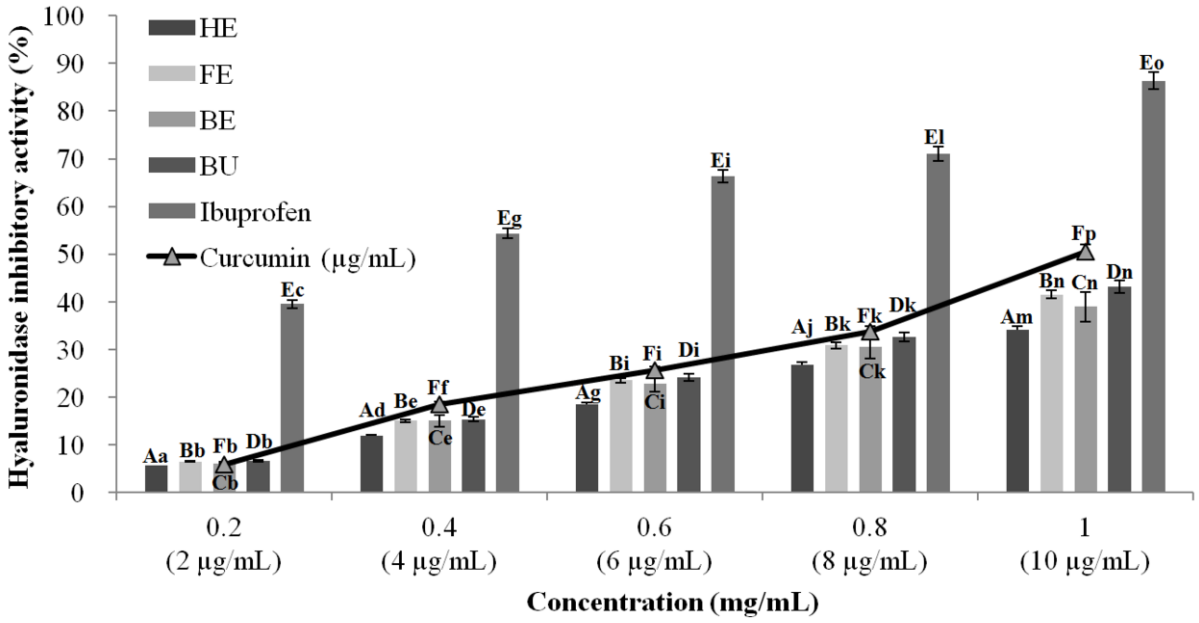

Fig. 2. Hyaluronidase inhibitory activities of the extracts of Curcuma longa leaf from several different drying methods.
*HE: Hot air drying extraction; FE: Freeze drying extraction; BE: Balanced low pressure drying extraction; BU: Balanced low pressure drying and ultrasonication extraction.
**Mean values±SD from triplicate independent experiments are shown. Means with difference letter (A-E) within the same sample are significantly different at $p<0.05$, and means with difference letter (a-o) within the same concentration are significantly different at $p<0.05$.

3.3.2. The production of Nitric Oxide (NO)

In order to skin immune activation associated with anti-inflammatory activities, the production of Nitric Oxide (NO) from macrophage RAW264.7 cells was observed [28,29], and Figure 3 showed the NO production in adding 0.25-1.0 mg/mL of the *C. longa* leaf extracts from various drying processes with and without LPS treatment. Treatment with LPS in cultivating macrophages cells produced only 8.2 μM of NO, which tells that LPS treatments were significantly induced an inflammatory reaction. For all types of extracts, the amounts of NO production decreased in a concentration-dependent tendency. Comparison in detail, macrophages produced 16.5 μM of NO after the addition of 1.0 mg/mL of BU, but only 15.5 μM of NO after the addition of 1.0 mg/mL of FE. BU and FE were shown slightly to inhibit NO generation; however, the BU was higher than that observed in the presence of BE (14.3 μM) and HE (13.2 μM) at 1.0 mg/mL concentration. The amounts of NO produced by macrophages treated with 0.25 mg/mL BU (12.7 μM) was higher compared to the amounts of NO generated by 1.0 mg/mL *Hericium erinacium* extract (9.2 μM) [30]. These results demonstrate that BU was the most active extracts in regulating induced-LPS nitrite production. In other words, the BU extraction was able to appear the skin immune activities high levels comparison to that of FE. Interestingly, camparison with curcumin (standard, 5 μg/mL), BU had the best activity in inhibiting nitrite production at 0.25 mg/mL followed by FE (11.9 μM), FE (10.8 μM), HE (10.7 μM) and curcumin (9.5 μM). Those results were the first report that improvement of only use stockfeed to high biological activities as like skin immune activities. The above results demonstrate that the skin immune activity of the *C. longa* leaf extracted using that balanced low pressure drying and ultrasonication extraction can increase the NO production to 16.5 μM at 1.0 mg/mL, which is higher than the other processes FE, BE

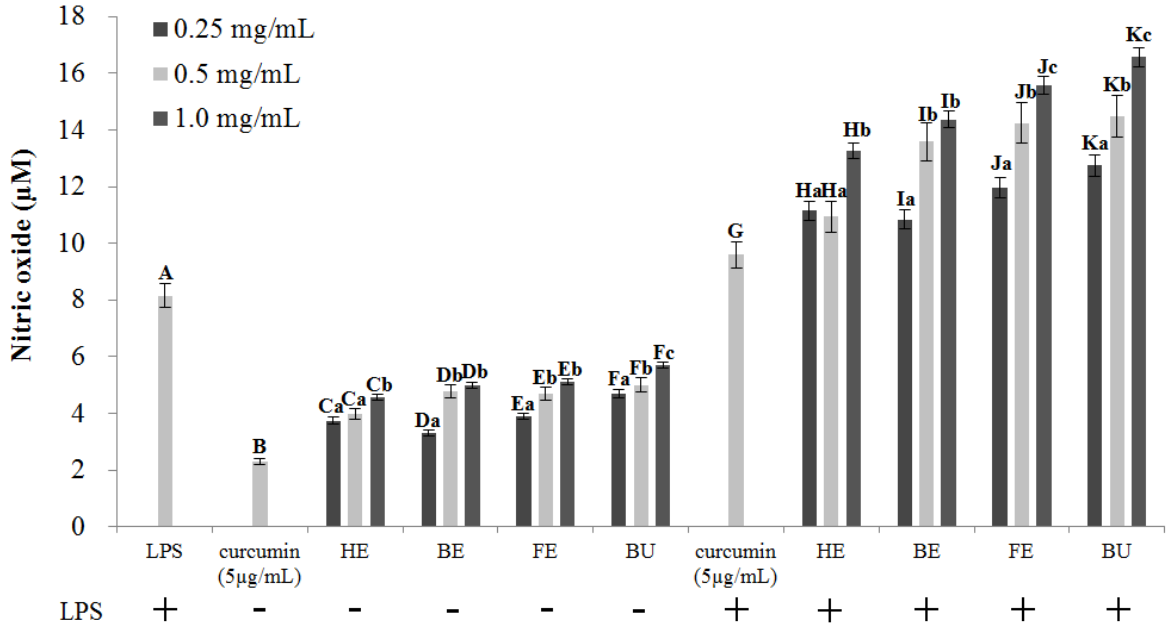

Fig. 3. Effect of the extracts of the Curcuma longa leaf on LPS-induced Nitric Oxide production from RAW264.7 cells.
*HE: Hot air drying extraction; FE: Freeze drying extraction; BE: Balanced low pressure drying extraction; BU: Balanced low pressure drying and ultrasonication extraction.
**Mean values±SD from triplicate independent experiments are shown. Means with difference letter (A-K) within the same sample are significantly different at $p<0.05$, and means with difference letter (a-c) within the same concentration are significantly different at $p<0.05$.

and HE. Therefore, this pretreatment extraction process (BU) significantly increases the skin immune activity.

3.3.3. Secretion of IL-6 and TNF-α from human T cell

To confirm the skin immune activities, the amounts of cytokines, which were related to skin inflammation [31], were observed in Figures 3 and 4. For the suppression of IL-6 production, as a control in treating LPS, 280% of IL-6 production increased compared to the case of non-treated cells (B in Figure 3) while in treating the extracts with extracts its production rate was noticeably inhibited. In treating 0.5 mg/mL of the extracts with LPS, the FE and the BU showed 50% and 53% IL-6 production, respectively, compared to the control, which means that the FE would be better in anti-inflammation at low concentration. However, in adding 2.5 mg/mL, the BU showed 63% production while the FE had 66 % possibly by much higher amounts of bioactive substances, curcumin in the BU at higher dosage than at lower dosage. Interestingly enough, for the case in adding the extracts without LPS, all of the BU showed better suppression of IL-6 production, and whose results indicate that the BU could play more effective role in controlling IL-6 secretion. Besides IL-6 secretion, Figure 4 also demonstrates the effect of the extracts on the inhibition of TNF-α expression, showing similar patterns of IL-6 secretion in Figure 3. Especially the extract from the FE showed minimum 82% of TNF-α expression in adding 2.5 mg/mL while the BU showed 62% of TNF-α production. Based on above results, TNF-α is considered that it induced the formation of immunity and anti-inflammation activities through formation of IL-1 and IL-6. At direct mechanism where cytokine IL-6

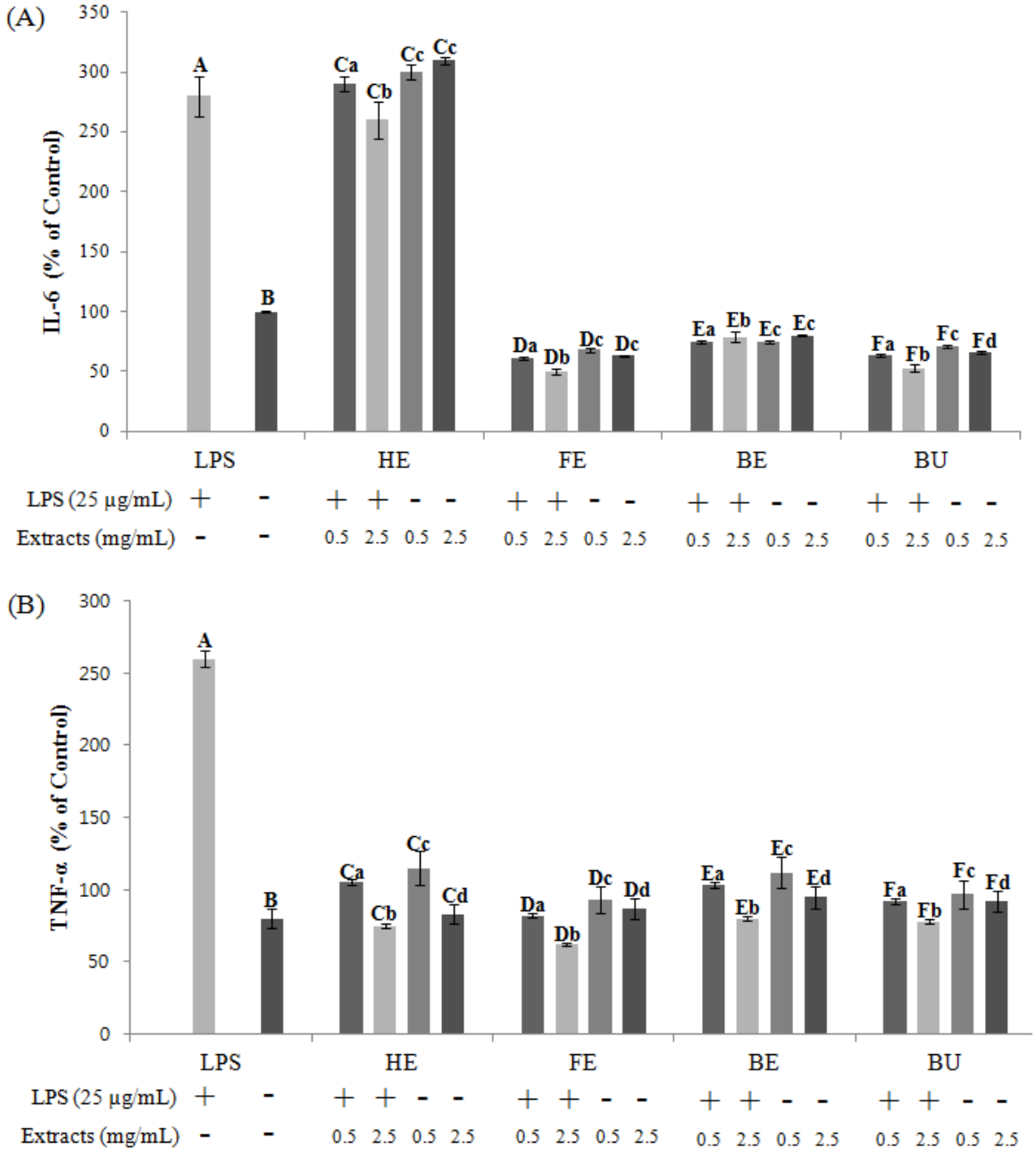

Fig. 4. Inhibitory activity of IL-6 and TNF-α production by the extracts from by several different drying methods.
*HE: Hot air drying extraction; FE: Freeze drying extraction; BE: Balanced low pressure drying extraction; BU: Balanced low pressure drying and ultrasonication extraction.
**Mean values±SD from triplicate independent experiments are shown. Means with difference letter (A-F) within the same sample are significantly different at $p<0.05$, and means with difference letter (a-d) within the same concentration are significantly different at $p<0.05$.

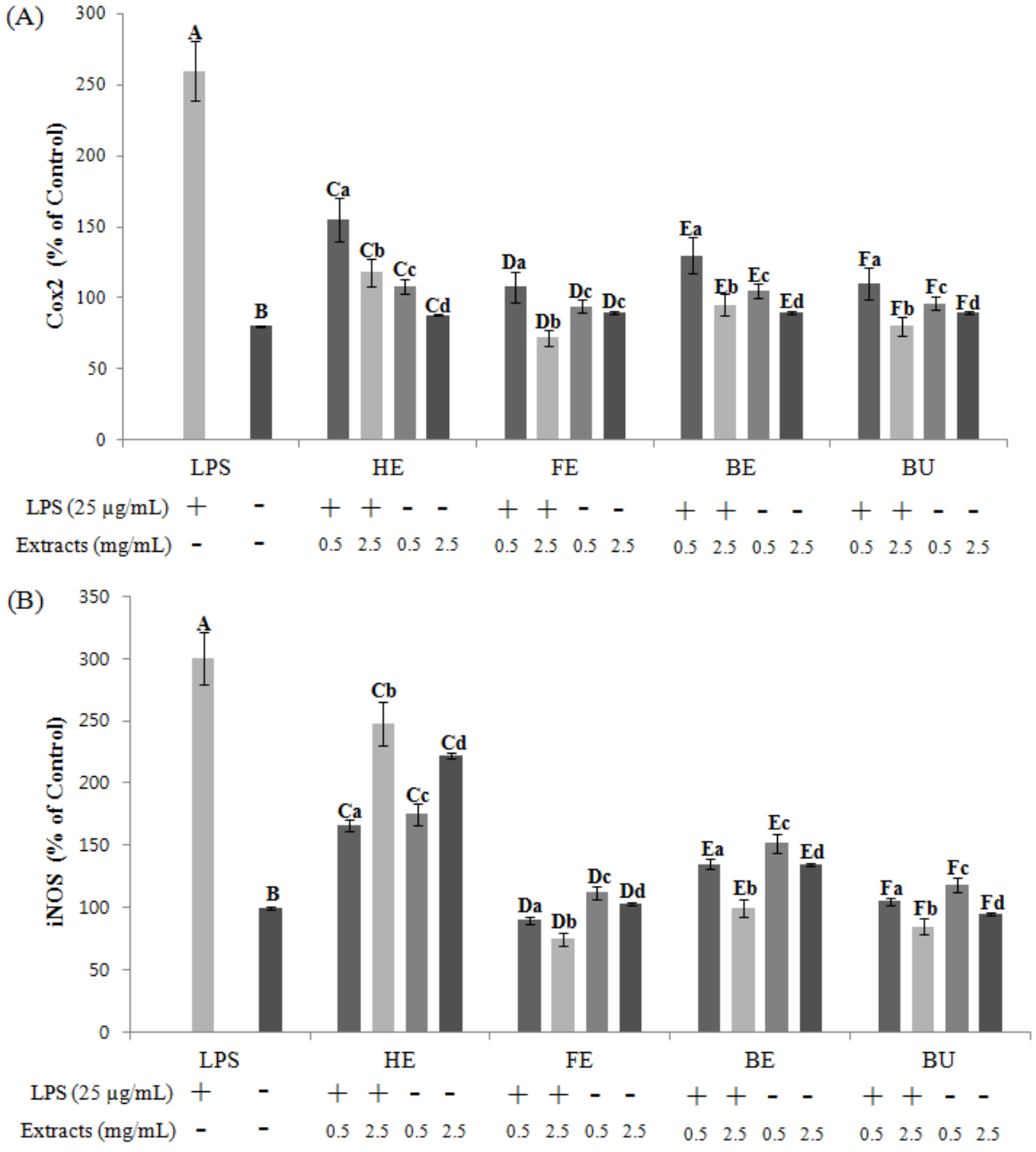

Fig. 5. Relative suppression of Cox-2 and iNOS gene expression by the extracts from several different drying processes.
*HE: Hot air drying extraction; FE: Freeze drying extraction; BE: Balanced low pressure drying extraction; BU: Balanced low pressure drying and ultrasonication extraction.
**Mean values±SD from triplicate independent experiments are shown. Means with difference letter (A-F) within the same sample are significantly different at $p<0.05$, and means with difference letter (a-d) within the same concentration are significantly different at $p<0.05$.

is produced, it is considered that the effect according to the expression of cytokine differed because precursor substance of IL-6 became rather unstable due to high temperature and the sample became partially damaged by high heat and hot air drying process [16]. It was also found that higher reduction of IL-6 and TNF-α production in comparing with the HE and BE while smaller reduction of the cytokines in comparing with the FE and the BU. This result indicates that better effects of the extracts could be expected in lower temperature processing than those considered in different extraction conditions. Thus, selecting pretreatment condition that can reduce the stimulation and minimize the damage on the natural substance should be prioritized for soft natural substances such as leaves instead of physical pretreatment process on a firm and thick natural substance such as stems and roots.

3.3.4. Suppression of Cox-2 and iNOS gene expression

To clearly understand the mechanism of skin anti-inflammation activities of the extracts, besides the inhibition of hyaluronidase inhibitory activity and cytokine production, the expression levels of Cox-2 and iNOS genes, which are closely related to anti-inflammation and immune activation [31], were measured for the case of an inflammation induction by addition of 25 μg/mL of LPS. Figure 5 shows that the expression of Cox-2 genes were greatly increased up to 260% of the gene expression compared to the control (not-treated cell) while iNOS was up to ca. 300% of the gene expression. Expression of Cox-2, which forms PGL2 for an immune system and inflammation, was inhibited overall by all of the extracts. Especially, the FE showed the maximum inhibitory level of 72% while the HE showed the least inhibitory level of 118%. However, the BU showed excellent suitability for thin leaf surface and its fragile properties by having 62% inhibition of Cox-2 expression, which would be similar or somewhat lower than that of the FE. For inhibiting of iNOS gene expression, which is a known gene for forming Nitric Oxide, showed 75% and 85% of high iNOS inhibition effect at high concentration of 2.5 mg/mL by the FE and the BU, respectively. Also, compared to the extracts from the HE or even the BE, the inhibition was 150% higher. These results shows that Cox-2 and iNOS gene expression were suppressed because of the bioactive substances related to immune system and anti-inflammation within formation mechanism of PGL2 and Nitirc Oxide, precursors of Cox-2 ad iNOS [23], and whose results were also well fit to the results of the amounts of curcumin, shown in Table 2.

4. Conclusion

It was proved that a relatively new drying method, balanced low pressure drying process could be economically feasible in drying weak parts of the medicinal plants like the leaves even though conceptually the freeze drying process would be most efficient, but less economical for the large scale processing. The balanced low pressure drying process was especially active on dehydration of the leaves with minimizing the destruction of the bioactive substances as well as making easier internal structures to be eluted, which resulted in higher extraction yield and biological activities. In this work, it was first found that the extraction yields and its biological activities of the leaves of *C. longa* were much influenced by drying temperature rather than the extraction conditions because the fragile structures of the leaves are more susceptible to the high drying temperature as well as generating high amounts of toxic residues. In general, the extracts from a balanced low pressure drying process associated with ultrasonication extraction at less than 40°C had higher skin anti-inflammation activities and lower cytotoxicity than that of freeze drying because the extract from EU contained higher contents of curcumin, and fewer amounts of toxic residue and less breaking down other bioactive substances due to high temperature processing. The elution of curcumin was greatly increased through

ultrasonication extraction process. It was observed that the extracts processed from the leaves of *C. longa* can efficiently suppress the expression level of inflammation related genes such as Cox-2 and iNOS as well as have skin anti-inflammation activities, which can prove the possibility of utilizing the byproducts of the medicinal plants.

Acknowledgement

This work was carried out with the support of Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ008435), Rural Development Administration, Republic of Korea.

References

[1] G.Y. Ryu, K.H. No, S.R. Ryu and H.S. Yang, Study of separation and analysis method an effective component from UlGeum (curcuma longa) and a contained curcumin as product of national and partialy region cultures, Appl. Chem. **9** (2005), 57–60.
[2] Y.B. Chae, K.T. Chung, S.G. Kim, B.H. Yoo and M.M. Kim, Effect of curcuma longa hot water extract on activity of neuronal cells related to oxidative stress, J. Life Sci. **22** (2012), 657–664.
[3] H. Taka, The characterization and application of Curcuma longa, L extracts, New Food Ind. **40** (1998), 7–15.
[4] R. Selvam, L. Subramanian, R. Gayathri and N. Angayarkanni, The anti-oxidant activity of turmeric (curcuma longa), J. Ethnopharmacol. **47** (1995), 59–67.
[5] H.P. Ammon and M.A. Wahi, Pharmacology of curcuma longa, Planta. Med. **57** (1991), 1–7.
[6] B. Gupta and B. Ghosh, Curcuma longa inhibits TNF-alpha induced expression of adhesion molecules on human umbilical vein endothelial cells, Int. J. Immunopharmacol. **21** (1999), 745–757.
[7] N. Shagufta, J. Safia, I. Saiqa, M. Farkhanda, A. Farah and A. Aamir, Antibacterial activity of Curcuma longa varieties against different strains of bacteria, Pak. J. Bot. **42** (2010), 455–462.
[8] D. Ahn, E.B. Lee, M.S. Ahn, H.W. Lim, M.M. Xing, Tao. C, J.H. Yang and D.K. Kim, Antioxidant constituents of the aerial parts of curcuma longa, Korean J. Pharmacogn. **43** (2012), 274–278.
[9] D. Ahn, E.B. Lee, M.S. Ahn, B.J. Kim, S.Y. Lee, T.G. Lee, H.W. Lim and H.Y. Lee, Flavonoids from the underground parts of curcuma longa, Korean J. Pharmacogn. **44** (2013), 253–256.
[10] J.S. Kim, Y.B. Choi, J.R. Ko and H.Y. Lee, Enhancement of biological activities of fresh ginseng by balanced low pressure drying processes, Chem. Eng. Prog. **17** (2013), 325–332.
[11] J.H. Hong and W.Y. Lee, Quality characteristics of osmotic dehydrated sweet pumpkin by different drying methods, J. Korean Soc. Food Sci. Nutr. **33** (2004), 1573–1579.
[12] J.S. Kim, J.W. Kim, H.S. Kwon, H.W. Lim and H.Y. Lee, Screening of skin whitening activity of Codonopsis lanceolata extract by complex steaming process, Korean J. Med. Crop Sci. **21** (2013), 54–60.
[13] Y.C. Seo, Y.B. Choi, J.R. Ko and H.Y. Lee, Quality characteristics of dried kimchi by cyclic low pressure drying, Food Eng. Prog. **17** (2013), 319–324.
[14] J.H. Park, H.S. Lee, H.C. Mun, D.H. Kim, N.S. Seong, H.G. Jung, J.K. Bang and H.Y. Lee, Effect of ultrasonification process on enhancement of immuno-stimulatory activity of ephedra sinica stapf and rubus coreanus miq, Korean Soc. Biotechnol. Bioeng. **19** (2004), 113–117.
[15] K.W. Chung, W.I. Kim, I.K. Hong and K.A. Park, Ultrasonic energy effects on squalene extraction from amaranth seed, Appl. Chem. **4** (2000), 149–152.
[16] W.I. Kim, K.W. Chung, S.B. Lee, I.K. Hong and K.A. Park, Ultrasound energy effect on solvent extraction of amaranth seed oil, J. Korean Ind. Eng. Chem. **13** (2001), 307–311.
[17] S. Himesh, P.S. Sharan, K. Mishra, N. Govind and A.K. Singhai, Qualitative and quantitative profile of curcumin from ethanolic extract of Curcuma long, Int. Res. J. Pharm. **2** (2011), 180–184.
[18] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J. Immunol. Methods **65** (1983), 55–63.
[19] J.L. Reissig, J.L. Strominger and L.F. Leloir, A modified colorimetric method for the estimation of N-acetylamino sugars, J. Biol. Chem. **217** (1955), 959–966.

[20] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok and S.R. Tannerbaum, Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids, Anal. Biochem. **126** (1982), 131–138.
[21] S.H. Oh, J.G. Han, J.H. Ha, Y. Kim, M.H. Jeong, S.S. Kim, H.S. Jeong, G.P. Choi, U.Y. Park, D.H. Kang and H.Y. Lee, Enhancement of immune activity of Spirulina maxima by low temperature ultrasonification extraction, Korean J. Food Sci. Tech. **41** (2009), 313–319.
[22] W.J. Yoon, J.A. Lee, K.N. Kim, J.Y. Kim and S.Y. Park, In vitro anti-inflammatory activity of the artemisia fukudo extracts in murine macrophage RAW 264.7 cells, Korean J. Food Sci. Tech. **37** (2007), 464–469.
[23] Y.S. Kim and J.M. Choi, Physiochemical properties and dyeability of safflower colorants extracted by ultrasonic treatment, J. Korean Soc. Cloth Ind. **11** (2009), 337–343.
[24] M.H. Jeong, S.S. Kim, J.H. Ha, Ling Jin, H.J. Lee, H.Y. Kang, S.J. Park and H.Y. Lee, Enhancement of anticancer activity of Acer mono by high pressure extraction process, J. Korean Soc. Food Sci. Nutr. **38** (2009), 1243–1252.
[25] M.C. Kwon, J.G. Han, J.H. Ahn, D.H. Lee and H.Y. Lee, Enhancement of immuno-potentiation of cichorium endivia L. by ultrasonification extraction process, Korean J. Med. Crop Sci. **16** (2008), 1–7.
[26] N.H. Lee, S.J. Lee, D.S. Jung, H.J. Bu, H.C. Yang and K.Z. Riu, Screening of the tyrosinase inhibition activities, and radical scavenging effects using plants in cheju, Korean J. Pharmacogn. **32** (2001), 175–180.
[27] S.I. Choi, Y.M. Lee and T.R. Heo, Screening of hyaluronidase inhibitory and free radical scavenging activity in vitro of traditional herbal medicine extracts, Korean Soc. Biotechnol. Bioeng. **18** (2003), 282–288.
[28] M.G. Cifone, S. Ulisse and A. Santoni, Natural killer cells and nitric oxide, Int. Immunopharmacol. **1** (2001), 1513–1524.
[29] J.R. Underwood, M. Chivers, T.T. Dang and P.V. Licciardi, Stimulation of tetanus toxoid-specific immune responses by a traditional chinese herbal medicine, Vaccine **27** (2009), 6634–6641.
[30] C.B. Lee, Anti-inflammation activity of water extracts from Hericium erinacium among medicinal mushrooms, Korean J. Culinary Res. **18** (2012), 233–242.
[31] O. Boyman, M. Kovar, M.P. Rubinstein, C.D. Surh and J. Sprent, Selective stimulation of T cell subsets with antibody-cytokine immune complexes, Science **311** (2006), 1924–1927.