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碩士學位論文

Physiological activities of Chestnut Flower extracts

朝 鮮 大 學 校 大 學 院

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밤꽃 추출물의 생리활성

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ABBREVIATIONS

BCA.....Bicinchoninic acid

BHT .....Butylated hydroxytoluene

BSA..... Bovine serum albumin

cDNA ..... Complement DNA

CF.....Chestnut flower

DEPC .....Diethyl pyrocarbonate

DMEM.....Dulbecco´s modified Eagle´s medium

DMSO..... Dimethyl Sulfoxide

dNTPs.....Deoxynucleic acid triphoshate

DPBS.....Dulbecco's Phosphate Buffered Saline

DPPH .....1, 1−diphenyl−2−picryl hydrazyl

EtBr .....Ethidium bromide

EDTA.....Ethylenediamine tetraacetic acid

FBS .....Fetal bovine serum

GAE .....Gallic acid equivalents

L−DOPA.....L−3,4−dihydroxyphenylalanine

M−MLV .....Moloney−murine leukemia virus ribonucleas

MOPs ..... 3-(N-morpholino) propanesultionic acid

MTT.. 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMR .....Nuclear magnetic resonance

OD .....Opticaldensity

PAGE ..... Polyacrylamide gel electrophoresis

PBS .....Phosphate buffered saline

PCR .....Polymerase chain reaction

PVDF .....Polyvinylidine difluoride

RT .....Reverse transcription

SDS ..... Sodium dodecyl sulfate

TEMED .....N,N,N',N'-tetramethylethylenediamine

QE ..... Quercetin

ABSTRACT

Physiological activities of Chestnut Flower extracts

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The antioxidant activities of chestnut flower extracts were examined using various antioxidant assays, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, reducing power. We investigated the effect of CF extracts on viability of normal and tumor cells by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Our results showed that CF extracts was not cytotoxic on mouse embryonic fibroblasts, NIH3T3. The viability of cells was increased significantly with low concentratins of CF extracts and conversely, it reduced cell proliferation and viability of most of the tested cancer cells (human lung cancer cell line, Calu6) in dose-dependent manners. However, CF extracts was not cytotoxic on human

hepatocellular carcinoma, SK-Hep-1.

Because tyrosinase catalyzes melanin synthesis, tyrosinase inhibitors are important in cosmetic skin-whitening. We examined CF extracts that might be useful for skin-whitening. CF extracts (30, 50 and 100 ㎍/㎖ each) were tested for cytotoxicity on human melanoma (SK-MEL-2). Their effects on tyrosinase and melanin inhibitory activities were further assessed. Results revealed that the prebloomed extracts exhibited the best performance in DPPH assay, reducing power assay. Furthermore, the preM has the highest total flavonoid contents (60.96 ± 1.86 ㎎ of QE/g) and preE has the highest total phenolic contents (467.92 ± 0.45 ㎎ of GAE/g). The results showed that CF extracts showed potent inhibitory effects on L-Dopa (L-3,4-dihydroxyphenylalanine) oxidation catalyzed by tyrosinase. CF extracts exhibited potent inhibitory effects on tyrosinase (preM 72.49%, preE 70.02%, preW 64.14%, postM 72.25%, postE 65.14% and postW 63.38% at 1 ㎎/㎖). Melanin inhibition was dose-dependent manner. In addition, we detected consistent downregulation of tyrosinase mRNA and proteins by CF extracts.

These results suggest that CF extracts have inhibitory effect on tyrosinase expression. The overall results of this study indicates that the extracts of chestnut flowers have interesting antioxidant properties and represent a potential source of medicine for the treatment of infectious diseases and cancer, tyrosinase inhibitor for cosmetic skin whitening.

Ⅰ. Introduction

Fruits and vegetables have been associated with the prevention of degenerative diseases, such as cancer and cardiovascular diseases (Liu, 2003). The presence of wide range of phytochemicals such as phenolics, thiols, carotenoids, anthocyanins and tocopherol have been suggested to exert chemopreventive (Dragsted, Strube & Larsen, 1993) and cardioprotective (Vita, 2005) effects, as well as protecting the human body against oxidative damage by free radicals (Halliwell, 1997).

Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. In healthy individuals, free radical production is continuously balanced by natural antioxidative defense systems (Gutteridge, 1993; Knight, 1995). Disruption of the balance between reactive oxygen species (ROS) production and elimination, to aging, leads to the process called oxidative stress. As a consequence, ROS are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, cataracts, chronic inflammation, and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Gutteridge, 1993; Knight, 1995). Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases (Halliwell, Gutteridge, & Cross, 1992). Thus, synthetic antioxidants are widely used in the food industry. However, because of their toxic and carcinogenic effects, their use is being restricted. Thereby,

interest in finding natural antioxidants, without undesirable side effects, has increased greatly (Rechner et al., 2002).

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase EC 1.14.18.1) is a multifunctional copper-containing enzyme present in microorganisms, plants and animals. This enzyme is mainly involved in the first two steps of melanin biosynthesis, which consist of the hydroxylation of L-tyrosine (monophenolase activity) and the oxidation of the product of this reaction, L-Dopa (diphenolase activity), to the corresponding O-quinone (Seo, Sharma, & Sharma, 2003) (Fig. 1). Tyrosinase is responsible for the pigment melanin biosynthesis in human skin. Clinically, various dermatological disorders, such as melasma, freckles and age spots, result in epidermal hyperpigmentation (Mosher, Pathak, & Fitzpatrick, 1983). Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in improving food quality and preventing pigmentation disorders and other melanin-related health problems in human beings (Maeda & Fukuda, 1991; Mosher et al., 1983; Seo et al., 2003). Furthermore, tyrosinase inhibitors are also important in cosmetics for skin-whitening effects because lighter.

Skin colour is preferred by many countries and races (Dooley, 1997). As plants are rich sources of bioactive chemicals, which are mostly free from harmful side-effects, there is an increasing interest in finding natural tyrosinase inhibitors from them. Some potent tyrosinase inhibitors, such as cuminaldehyde (Kubo & Kinst-Hori, 1988), oxyresveratrol (Shin et al., 1998), kaempferol (Kubo & Kinst-Hori, 1999), quercetin (Chen & Kubo, 2002) and gallic acid

derivatives (No et al., 1999) have been isolated from various plants. In addition to higher plants, a fungal metabolite, kojic acid [5-hydroxy-2-(hydroxymethyl)-r-pyrone], has been demonstrated to be a potent tyrosinase inhibitor and is extensively used as a cosmetic agent with an excellent whitening effect (Chen et al., 1991b; Kahn, Ben-Shalom, & Zakin, 1997). Plants play a significant role in maintaining human health and improving the quality of human life. They serve humans well as valuable components of food, such as seasonings and beverages as well as in cosmetics, dyes, and medicines. In fact, many plant extracts prepared from plants have been shown to exert biological activity *in vitro* and *in vivo*. A large number of plants have been screened as a viable source of natural antioxidants including tocopherols, vitamin C, carotenoids and phenolic compounds which are responsible for maintenance of health, to help the human body reduce oxidative damage and protection from coronary heart diseases and cancer (Yanga et al., 2002). There has been increasing realization in recent years that several plant derived polyphenolic compounds may possess antimicrobial, antioxidant, anticancer and apoptosis inducing properties (Clement et al., 1998). Therefore, the role of plant derived polyphenols in chemoprevention of cancer has emerged as an interesting area of research. To date, many anticancer drugs have been developed and applied by clinical doctors. However, the resistance to cancer drugs and side effects were discovered (Yang et al., 2000).

Chestnut (*Castanea crenata var. dulcis*) flower is a traditional herbal medicine used diarrhea and hema feces (Hirokawa et al., 1986). The flowers follow the

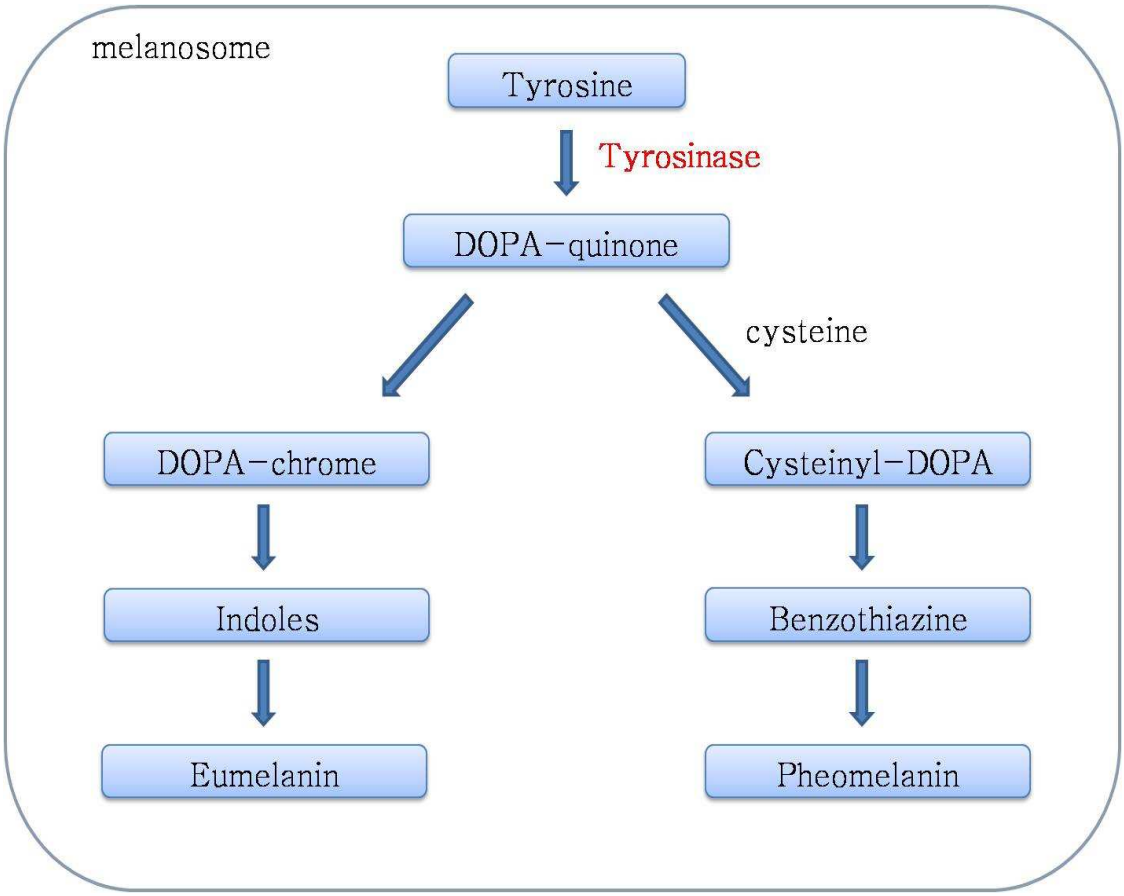

Fig. 1. Melanin synthetic pathway

leaves, appearing in late spring or early summer. They are arranged in long catkins of two kinds, with both kinds being borne on every tree (Fig. 2). Although it has already been demonstrated that chestnut fruits (Ribeiro et al., 2007) and leaves (Calliste et al., 2005) contain phenolic compounds, little is known about their antioxidant potential or about other chestnut extracts, such as skins and flowers. Furthermore, it is considered to have a skin-whitening effect. However, no information is available about the effects of chestnut flower against mushroom tyrosinase activity.

Accordingly, in this study, the antioxidant properties, anticancer, tyrosinase inhibition activity and melanin synthesis contents of chestnut flower extracts were evaluated through several biochemical assays.

Fig. 2. Prebloomed and postbloomed Chestnut flower

Ⅱ. Materials and methods

A. Materials

For determination of free radical activity and antioxidant capacity, 1,1-diphenyl-2picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT) and Ascorbic acid (vitamin C), Gallic acid, Quercetin were purchased from sigma-Aldrich (U.S.A.).

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, Fetal bovine serum (FBS), Dulbecco's Phosphate-Buffer Saline (DPBS), TrypLE™ Express (Trypsin-EDTA solutions) were purchased from GIBCO BRL (U.S.A.). Dimethylsulfoxide(DMSO),3-(4,5dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) were purchased from sigma-Aldrich (U.S.A.). Tissue culture dishes, flasks, multiplex 96 wells plates, 12 well plates were purchased from the Nunc (Denmark).

Tyrosinase mushroom (5370 units/㎎), L-3,4-dihydroxyphenylalanine (L-Dopa) were purchased from Sigma-Aldrich (U.S.A.).

For RT-PCR, Trizol™ was purchased from the Invitrogen Co. (U.S.A.). Isopropanol, chloroform, diethyl pyrocarbonate (DEPC), 3-(N-morpholino) propanesulfionic acid (MOPs), formamide, formaldehyde, and ethidium bromide (EtBr) were purchased from the Sigma-Aldrich Co. (U.S.A.). Agarose was purchased from the Seakem® Cambrex (U.S.A.). Moloney-murine leukemia virus ribonuclease (M-MLV), oligo dT (deoxythymidine) primer, dNTPs

(deoxynucleic acid triphosphate), Taq polymerase, specific primers (for β-actin, Tyrosinase), and 100 bp DNA ladder were purchased from the BioNEER Co. (Korea).

B. Methods

1. Sample extraction

a. Preparation of Chestnut flower extract

Three types of extracts (ethanol, methanol and water extract) were prepared as a testing samples. Prebloomed chestnut flower and postbloomed chestnut flower (500 g each) were extracted with 1,500 ㎖ of 70% methanol, 70% ethanol for 10 days at room temperature. The sample was extracted using 1,500 ㎖ of water at boiling temperature for 2 hr. The extracts were filtered through Whatman filter paper (110 ㎜ Ø) and the solvents were vacuum-distilled at 40℃ in a rotary evaporator. The ethanol (CFE), methanol (CFM) and water (CFW) extracts were freeze-dried and used in this study as appropriate to the each experiments soluble part. After concentration, the concentrate was centrifuged to remove insoluble materials.

b. Analysis of total flavonoid compounds

The total flavonoid compounds were measured using the method of Maria (2000). Flavonoids concentration was determined as follows: CFM, CFE and CFW (100 ㎕) were diluted with 80% aqueous ethanol (900 ㎕). An aliquots of 100 ㎕ were added to EP tubes containing 20 ㎕ of 10% aluminum nitrate, 20 ㎕ of 1 M aqueous potassium acetate and 860 ㎕ of 80% ethanol. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 ㎚. Total flavonoid concentration was calculated using quercetin as standard (Park *et al.*, 1997). The experiment was repeated three times.

c. Analysis of total phenolic compounds

The total phenol contents of each extract were determined using the Folin-Ciocalteu reagent (Zhou & Yu, 2006) with some modifications. Briefly, stock solutions were prepared by dissolving 1 ㎎ of extracts in 1 ㎖ water. These stock solutions (25 ㎕) was added to 500 ㎕ of 0.1 × Folin-Ciocalteau regent in the EP tube. After 5 min at room temperature, 500 ㎕ of Na_2CO_3 (7.5% w/v) solution were added and the mixture was allowed to stand for 90 min with intermittent shaking. After 90 min, absorbance was measured at 725 ㎚ using Pharmacia Biotech UV/Visible spectrophotometer (Ultrospec 2000). The total phenolic content was expressed as gallic acid equivalents (GAE) in ㎎ per g extract. The experiment was repeated three times.

2. Determination of DPPH radical scavenging activity

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured according to the method of Blois (1958) with minor modifications. The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant. Samples were dissolved in absolute methanol and then centrifuged to remove insoluble materials. Briefly, 100 ㎕ of 0.4 mM DPPH in methanol was mixed with 10 ㎕ of extract solution with differing concentrations (10, 50, 100, 300 and 500 ㎍/㎖). The 96 well plate was allowed to stand at room temperature for 30 min. A control was prepared as described above without CFM, CFE and CFW or standards. Methanol was used for baseline correction. The changes in the absorbance of the all the samples and standards were measured at 517 ㎚. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. L-ascorbic acid (Vitamin C) and 2,6-Di-tert-butyl-4-methylphenol (BHT) were used standard. The radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

3. Reducing power assay

The reducing power from CFM, CFE and CFW were determined according to methods of Oyaizu (1986). Briefly, 200 ㎕ of CFM, CFE and CFW at various concentration (10, 50, 100, 300, 500 and 1000 ㎍/㎖) were mixed with 200 ㎕ of 0.2 M sodium phosphate buffer (pH 6.6) and 200 ㎕ of potassium-ferricyanide (1% w/v). The mixture was incubated at 50℃ for 20 min and 600 ㎕ of 10% trichloroacetic acid were added. After centrifugation for 10 min at 1500 g, 500 ㎕ of the supernatant was added to the tubes containing 500 ㎕ distilled water and 100 ㎕ 0.1% $FeCl_3 \cdot 6H_2O$. The absorbance of resulting solution was measured at 700 ㎚ using water as blank. A control was also prepared replacing water with extract. Ascorbic acid which possesses relatively strong reducing power was used as a standard and reducing power was expressed as % ascorbic acid.

4. Enzymatic assay of tyrosinase

The tyrosinase activity, using L-DOPA as substrate, was measured according to the method of Yagi (1986) with slight modifications. First, the extracts (1, 1.5 and 2 ㎎/㎖) were weighed and dissolved in 0.175 M phosphate buffer (pH 6.8). Then, 50 ㎕ of sample was added to 10 ㎕ of 0.175 M phosphate buffer (pH 6.8), 20 ㎕ of 110 U/㎖ mushroom tyrosinase and 20 ㎕ of DOPA (5 mM) in a well of a 96 well plate. The samples were incubated for 20 min at 25℃. Absorbance was measured at 475 ㎚. Inhibition effects on the enzyme activity by test samples

were represented as % of inhibition.

$$\text{Tyrosinase inhibition (\%)} = [\,(A - B) / A\,] \times 100$$

A : ΔOD_{475} without test sample

B : ΔOD_{475} with test sample

5. Cell culture and treatment

Cell strains used in this study were NIH3T3 (Mouse embryonic fibroblasts), Calu6 (Human lung cancer), SK-Hep-1(Human hepatocellular carcinoma), SK-MEL-2 (Human melanoma). All strains were obtained from the Korean cell line bank.

Mouse embryonic fibroblasts cell line NIH3T3, human hepatocellular carcinoma cell line SK-Hep-1 were cultured in DMEM. Human lung cancer cell line Calu6, human melanoma cell line SK-MEL-2 were cultured in RPMI 1640 medium. All of these media were supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 units/㎖ penicillin, and 100 ㎍/㎖ streptomycin sulfates. All these cells were maintained in a humidified incubator under 5% CO_2 at 37℃. The cells were routinely subcultured twice a week.

For experimented cells were seeded at 1×10^5 cells/well and left for 24 hr prior to experimental treatments. Normal cells (30, 50, 100, 300 and 500 ㎍/㎖)

and cancer cells (30, 50 and 100 ㎍/㎖) were treated with different concentrations of CF extracts for 24 hr to investigated the cell viability.

a. Determination of cell viability

The evaluation of cytotoxic activity is based on the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) by the mitochondrial dehydrogenase of viable cells to give a blue formazan product which can be measured spectrophotometrically, performed with a slight modified method from Moongkarndi et al., (2004).

Cells were first cultured in T-75 culture flasks. The MTT colorimetric assay was performed in 96 well plates. After cells grown up to 70% confluence, cells were seeded in 96 well plates at a concentration of 1×10^5 cells/well and incubated during 24 hr at 37℃ with 5% CO_2. The various concentrations of each extract of CF were added to the cultures for 24 hr at 37℃. At the end of the incubation, 10 ㎕ of MTT reagent (final concentration is 0.5 ㎎/㎖) in medium without FBS was added into each well and incubated at 37℃ for 4 hr. The medium was removed and then DMSO 100 ㎕ was added into each wells to dissolve the formazan crystal. The absorbance was measured at 540 ㎚ using VERSAmax Microplate Reader (Molecular Devices, U.S.A.). Cell viability was calculated as the percent ratio of absorbance of the samples to the referent control.

$$\text{Inhibitory rate (\%)} = ((OD_{control} - OD_{sample})/OD_{control}) \times 100$$

b. Melanin content determination

Estimations of melanin content were performed using a modified method of Bilodeau et al., (2001). SK-MEL-2 cells (1×10^5 cells/㎖) were seeded in 12 well plates. Cells were then incubated for 48 hr with or without extract at concentrations (30, 50 and 100 ㎍/㎖). After washing twice with PBS, samples were dissolved in 400 ㎕ of 0.2 N NaOH. The samples were incubated at 60℃ for 1 hr and mixed to solubilize the melanin. Absorbance at 405 ㎚ was compared with a standard curve for synthetic melanin.

c. Total RNA extraction

Total RNA was extracted from SK-MEL-2 cells using TRIzol reagent (Invitrogen, Life Technologies, U.S.A.) following the protocol provided by company, which is based on the method described by Chomczynski (1993). After havesting, cells were lysed in TRIzol reagent by repetitive pipetting. Homogenization was followed by phase separation by addition of 200 ㎕ of chloroform and centrifugation at 12,000 ×g at 4℃ for 15 min. The aqueous phase was then transferred to a centrifuge tube containing 500 ㎕ isopropanol

and incubated at room temperature for 10 min. The resulting mixture was then centrifuged at 12,000 ×g at 4℃ for 10 min. After being washed with 75% ethanol the visible RNA pellet was dried and suspended in a small volume of DEPC-treated water. The RNA concentration was determined by optical density (OD) at 260 ㎚. Integrity of the RNA was verified by gel electrophoresis of 4 ㎍ RNA on a 0.8% agarose gel containing 2.2 M formaldehyde. Isolated intact samples were visualized both 28S and 18S (ribosomal RNA) bands. Samples were stored at −20℃ until subsequent analyses.

d. Reverse transcription (RT)-Complement DNA (cDNA) preparation

Total RNA samples were reverse transcribed for each sample to be analyzed by incubation with a reverse transcription mixture containing the following constituents : 10 pmol oligo (dT) primer, 5 × PCR buffer, 0.1 M DTT, 10 mM dNTPs, 20 units of RNase inhibitor, and 200 units of Moloney-murine leukemia virus ribonuclease (M-MLV). The reaction mixture was incubated for 60 min at 42℃ followed by 5 min at 70℃ inactivate the RT enzyme. Quality of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at −20℃ for further using.

e. Polymerase chain reaction (PCR) and the analysis of PCR products

The cDNA in the RT product was amplified using Taq DNA polymerase. PCR reaction was performed in 20 ㎕ of the total volume using 10 pmol of corresponding primers (Table 1). The cDNA was amplified under the following reaction conditions: denaturation at 94℃ for 30 sec for β-actin, for 2 min for

Table 1. List of primers used for RT-PCR

Genes	Primer sequences	Nucleotides Position	PCR product	Gene reference
β-actin	Forward : CCTCTATGCCAACACAGT Reverse : AGCCACCAATCCACACAG	No. 957~1111	155bp	BC063166
Tyrosinase	Forward : TTAGCAAAGCATACCATCAG Reverse : GAGAGACATTGATTTTGCCC	No. 920~1106	187bp	M27160

tyrosinase, annealing at 56℃ for 30 sec for β-actin, at 61℃ for 1 min for tyrosinase, polymerization at 72℃ for 30 sec for β-actin, for 1 min for tyrosinase. The cyclic process was performed 25 times for β-actin and 35 times for tyrosinase. PCR products were analyzed on 1.2% agarose gel and visualized by EtBr. Staining intensity of individual bands was evaluated by Gel Quant software (DNR Bio-Imaging Systems Ltd.).

f. Total protein isolation and protein concentration determination

For isolate proteins, we were harvested cells using scraper. The cell pellets were lysed for 30 min at ice in RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). After incubation, lysates were centrifuged for 15 min at 13,000 ×g at 4℃. The supernatants was used as protein fractionation

Protein concentration was measured using a BCA protein assay kit (Pierce Co., U.S.A.). BSA (2 ㎍/㎕) was used as the standard. 50 parts of BCA solution A was mixed with 1 part of BCA solution before using 10 min. BSA (0, 10, 20, 30 and 40 ㎍/㎕) and 2 ㎕ of each sample was inserted into 96 well plate wells. 200 ㎕ of the working reagent was added to each well. Plate was covered with foil and incubated for 30 min at room temperature in the dark. The absorbance was measured at 562 ㎚ on Versamax Microplate Reader (Molecular Devices, U.S.A.).

g. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is based on separation of a large range of proteins of varying molecular weights and charges under the influence of the electrical field within the continuous cross-linked polymer matrix polyacrylamide/ bis-acrylamide (Rothiphorese). Cross-linking is catalysed by free radicals produced upon addition of ammonium peroxide and TEMED. Two-phase gels were used for collection and separation of the proteins according to their molecular weight. Protein samples (20 μg/lane) were separated by SDS-PAGE with a miniprotein Ⅱ apparatus (Bio-Rad, U.S.A.) was used. Electrophoreses were run on polyacrylamide gels (5% stacking and 10.5% separating) with a migration buffer consistion of a 0.05 M tris-HCl (pH 8.8), 0.4 M glycine and 0.1% SDS solution. The samples were diluted in a sample buffer consisting of a 60 mM tris-HCl (pH 6.8), 25% glycerol, 14.4 mM β-mercaptoehanol, 0.1% bromophenol blue and 2% SDS solution. To define a molecular weight of loaded proteins the molecular weight marker was loaded and separated in parallel. Equal volumes of protein samples and 2× SDS-loading buffer were mixed, subjected to heating at 95℃ for 5 min, chilled on ice and loaded in the gel combs. SDS-PAGE was run at 4℃ in ice-cold electrophoresis buffer. Electric field of 70 V was applied for 15 min to allow samples to enter the gel and collect without a smearing and then increased to 100 V and kept constant until the bromophenol blue reached the bottom of the resolving gel.

h. Western blot analysis

After SDS electrophoresis, Proteins were transferred to PVDF membrane for 2 hr at 4℃. Membrane was blocked with 3% BSA in TBS-T for overnight at 4℃. Primary antibody was used for 2 hr at 4℃ and secondary antibody was used for 2 hr at room temperature. The membrane was washed 6 × 5 min with TBS-T between primary and secondary antibody. After incubation with secondary antibody conjugated with HRP, the membrane was washed 4 × 5 min with TBS-T. To reveal the reaction bands, the membrane was reacted with WEST-ZOL (plus) Western blot detection system (Intron Biotechnology, Inc., Korea) and exposed on X-ray film (BioMax MS-1, Eastman Kodak, U.S.A.). A digital image system was used to determine the density of the bands (Gel Quant, DNR Bio-Imaging Systems Ltd., U.S.A.).

6. Statistical evaluation

Data were analyzed using the Sigma Plot software. All the experiments were determined in triplicate and repeated three times to ensure reproducibility. Data were expressed as mean ± standard errors of mean (S.E.M.) of three independent experiments. Results were evaluated by Student's t-test or by analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered significant.

Ⅲ. RESULTS

A. Antioxidant activities in CF extracts

1. Analysis of total flavonoid and phenolic compounds

As one of the most important antioxidant plant components, phenolic compounds are widely investigated in many medicinal plant and vegetables (Djeridane et al., 2006). Although most antioxidant activities from plant sources are derived from phenolic-type compounds (Cai, Luo, Sun & Corke, 2004), these effects do not always correlate with the presence of large quantities of phenolics. Therefore, both sets of data need to be examined together. For this, the extracts were analysed for total phenolic and flavonoid contents. The total phenolic compounds and the total flavonoids were measured for all the samples. Total phenolic compounds were found in all the samples and in the following order : preE > preM > preW > postM > postE > postW. The content of phenolic compounds in extracts, expressed in gallic acid equivalents (GAE), varied between 251.62 and 467.92 ㎎/g. The results given in Table 2 shown that the highest content of total phenolic compounds was found in extracts (467.92 ± 0.45 mg/g in preE extract). Total flavonoid compounds were in the following order : preM > preE > postE > postW > postM > preW. The CF extracts had high total flavonoid compounds (60.96±1.86, 41.59±8.57, 26.75±1.44, 27.93±0.84, 31.60±1.77, 30.04±2.78) than other plant extracts (N. Ozsoy et al., 2008, Ji-

Table 2. The total flavonoid compounds, phenolic compounds contents

Sample		Flavonoid compound (mg QE/g)	Phenolic compound (mg GAE/g)
Prebloomed	Methanol extract	60.96 ± 1.86	456.24 ± 5.88
	Ethanol extract	41.59 ± 8.57	467.92 ± 0.45
	Water extract	26.75 ± 1.44	350.52 ± 3.39
Postbloomed	Methanol extract	27.93 ± 0.84	327.17 ± 7.09
	Ethanol extract	31.60 ± 1.77	313.14 ± 1.38
	Water extract	30.04 ± 2.78	251.62 ± 0.79

Young Kim et al., 2007).

2. Determination of DPPH radical scavenging activity

The role of antioxidant is removal of free radicals. One mechanism through which this is achieved involves donating hydrogen to a free radical and hence its reduction to an unreactive species. Addition of hydrogen removes the odd electron feature which is responsible for radical reactivity (Hua et al., 2008). All the extracts were capable of scavenging DPPH radicals in a concentration-dependent manner. Fig. 3 shows the dose-response curves of DPPH radical scavenging activities of CF extracts. CF extracts had stronger scavenging activity on DPPH radial than BHT. This activity was preM with 53.73±0.27%, preE (53.68±0.45%), postM (37.55±0.7%), postE (33.46±0.53%), postW (23.19±0.31%), preW (21.57±0.38%), and BHT with 2.34±0.2%, which were greater than that of ascorbic acid (vitamin C) with 92.94±0.53% at the level of 50 ㎍/㎖ ($p<0.05$).

3. Determination of reducing power

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003). Fig. 4 shows the dose-response curves for the

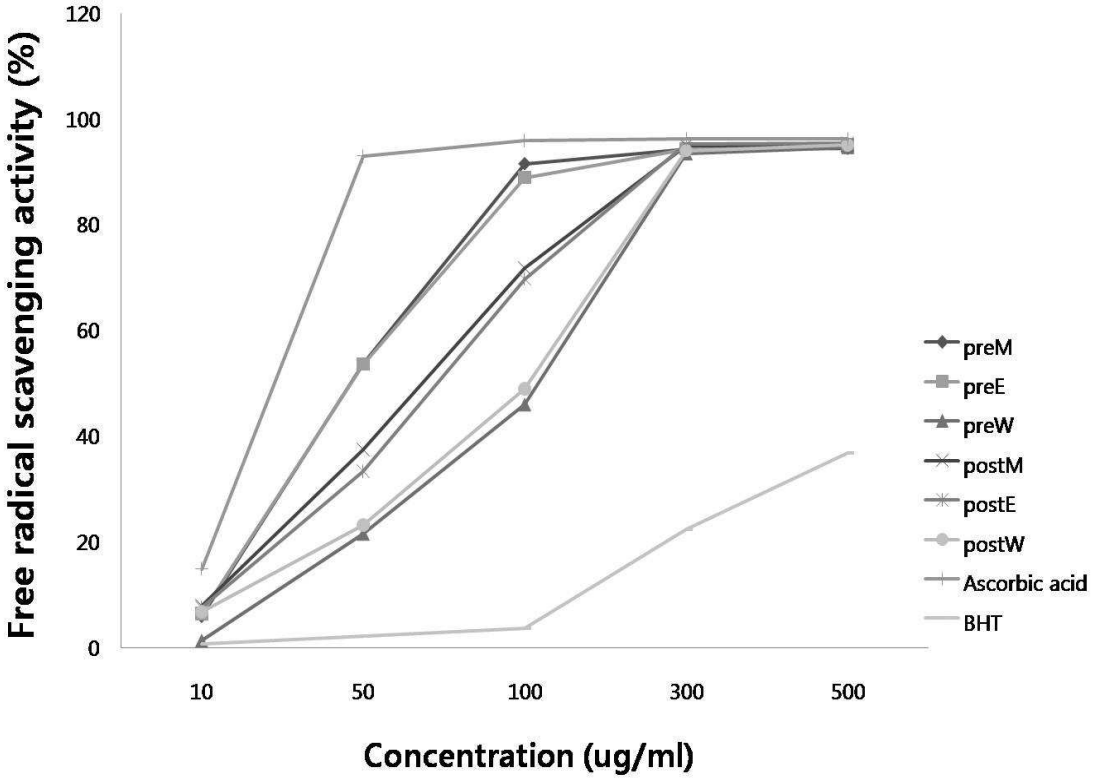

Fig. 3. Effects on DPPH radical scavenging activity of CF extracts were compared with that of ascorbic acid (Vit. C) and that of Butylhydroxytoluene (BHT).

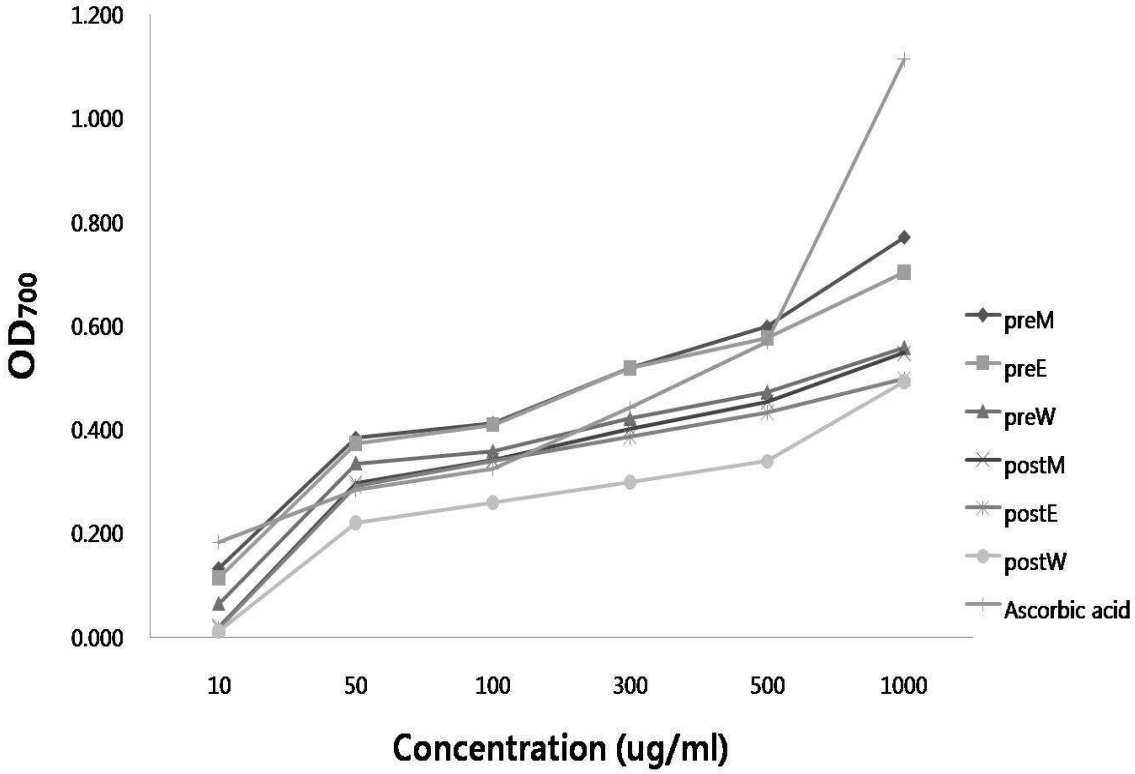

Fig. 4. The reducing power of ascorbic acid and CF extracts. CF extracts were compared with that of ascorbic acid (Vit. C).

reducing powers (RP) of the extracts from CF. The reducing powers of the samples were found to be in the following order : ascorbic acid (0.184−1.115) > preM (0.133−0.772) > preE (0.115−0.704) > preW (0.065−0.559) > postM (0.02−0.548) > postE (0.017−0.498) > postW (0.012−0.494). Ascorbic acid only showed slightly higher activity with a reducing power of 1.115±0.02 at 1 ㎎/㎖.

The published the reducing power in the other *Kappaphycus alvarezii* (methanol, ethanol, ethyl acetate, water and hexane were 0.07−0.074, 0.333−0.44, 0.013−0.467, 0.017−0.193 and 0.017−0.16 at 0.5 to 5.0 ㎎/㎖) (Suresh et al., 2008) and *Monascus* fermented soybeans (MFS)−31499 and MFS−31527 (cold water extracts and hot water extracts were both 0.54 at 5 ㎎/㎖ and 0.48 and 0.31 at 5 ㎎/㎖) (Lee et al., 2008).

B. Effects of CF extracts on viability of normal cells

The loss of cell viability in culture is generally measured by the reduction of MTT activity. The effect of CF extracts on cell viability was determined by MTT using NIH3T3, which is originated from mouse fibroblast. Fig. 5 shows the effects of CF extracts on cell viability of NIH3T3 cells after incubating for 24 hr with different concentrations using the MTT assay. The viability of NIH3T3 cells increased significantly by 9% with 50 ㎍/㎖ postE after 24 hr incubation. However, when 100 ㎍/㎖ of prebloomed extracts was added to NIH3T3 cells for 24 hr, less than 30~50% of cell viability was observed, compared to the

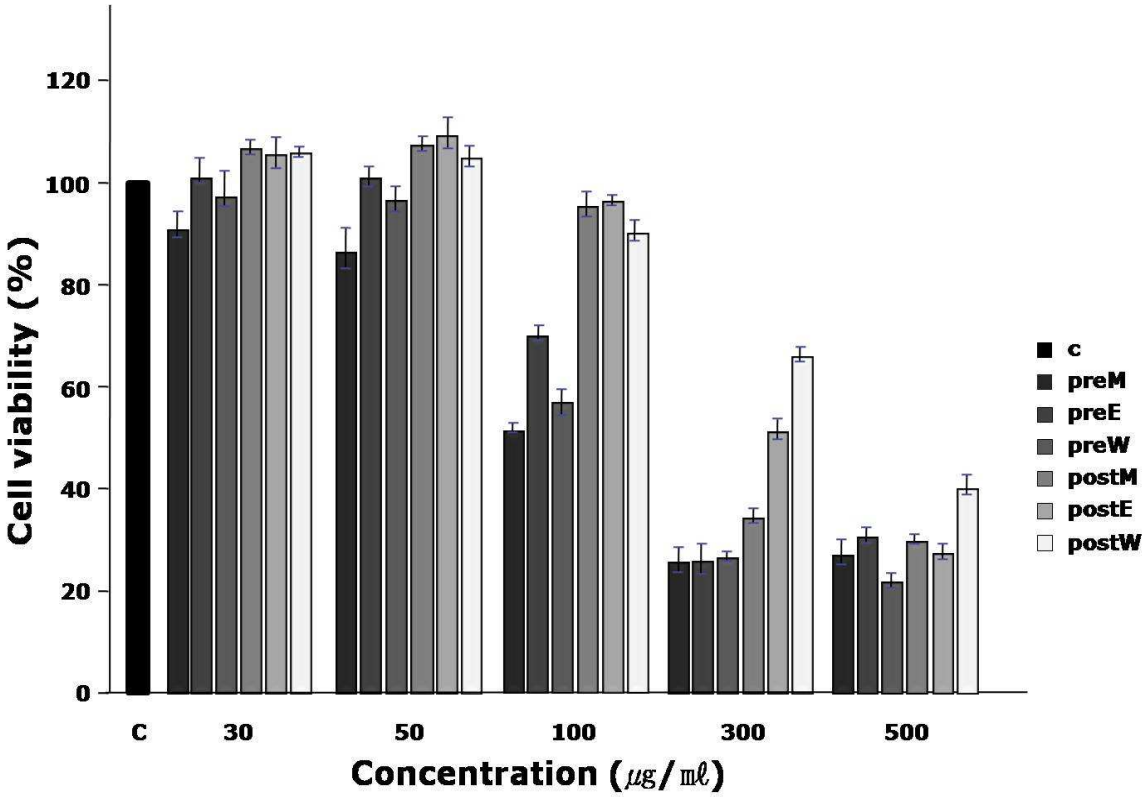

Fig. 5. Effects of CF extracts on cell viability of mouse fibroblast cell line, NIH3T3.

Cells were treated with the indicated concentrations of CF extracts for 24 hr. Levels of the cell viability were measured using the MTT assay. The viability of untreated control cells was defined as 100%. Results shown are means ±SD (n=3).

untreated cells.

C. Effects of CF extracts on cytotoxicity to cancer cells

To characterize the mechanism by which CF extracts inhibits the proliferation of cancer cells, we attempted to ascertain whether CF extracts exerted a cytotoxic effect on different cancer cells using a MTT assay, in which a colorless tetrazolium salt is cleaved and converted to a blue formazan by mitochondrial dehydrogenases of living cells.

Two types of human cancer cells; Calu6 (Human lung cancer), SK-Hep-1 (Human hepatocellular cacinoma) were exposed to different concentrations of CF extracts for 24 hr. Fig. 6 and Fig. 7 shows the effects of CF extracts on cell cytotoxicity of Calu6 and SK-Hep-1 cells after incubating for 24 hr with different concentrations using the MTT assay.

As shown in Fig. 6, the MTT assay shown that the addition of CF extracts reduced the viability of Calu6 cells in a dose-dependent manner. When 50 ㎍/㎖ of the CF extracts was added, less than 50% of cell viability was observed, compared to that of untreated cells.

In the human hepatocellular carcinoma cells SK-Hep-1, the cytotoxic effect of CF extracts on cell viability was not changed up to a concentration of 30 ㎍/㎖ even after 24 hr incubation (Fig. 7). However, following a 24 hr incubation of the SK-Hep-1 cells with 100 ㎍/㎖ of postW, cell viability was found to be 94%.

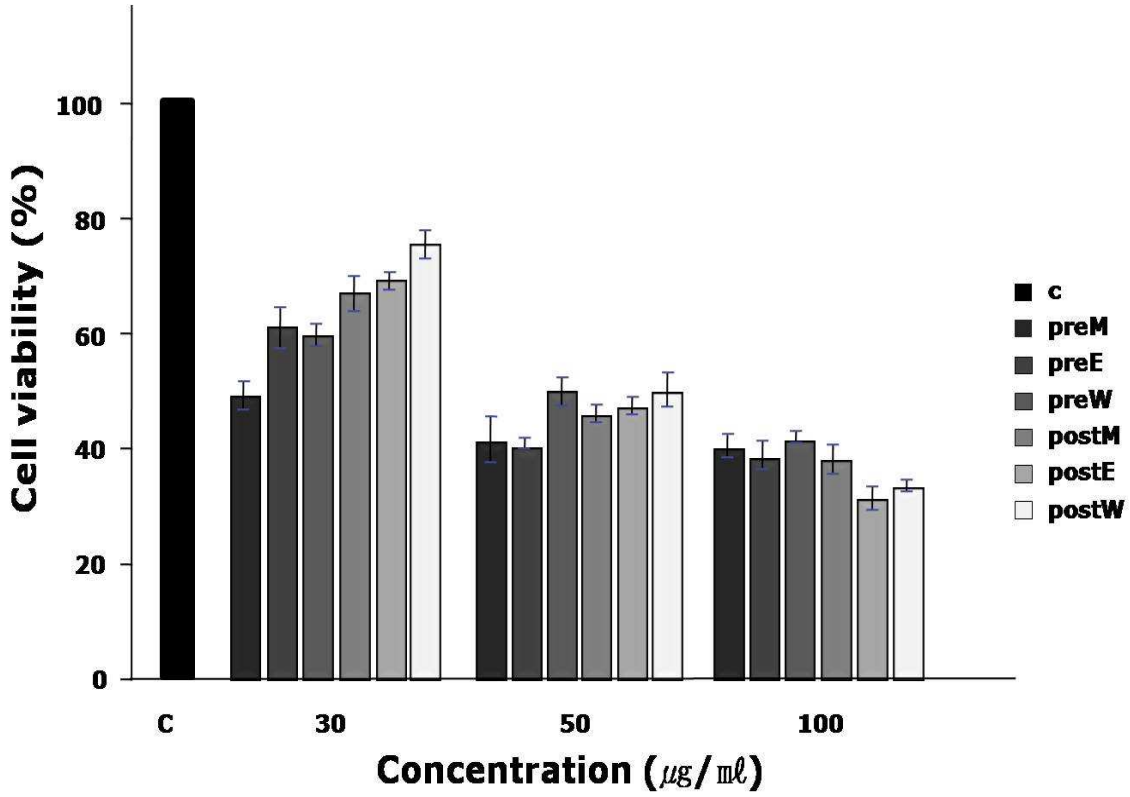

Fig. 6. Effects of CF extracts on cell viability of human lung cancer cell line, Calu6.

Cells were treated with the indicated concentrations of CF extracts for 24 hr. Levels of the cell viability were measured using the MTT assay. The viability of untreated control cells was defined as 100%. Results shown are means ±SD (n=3).

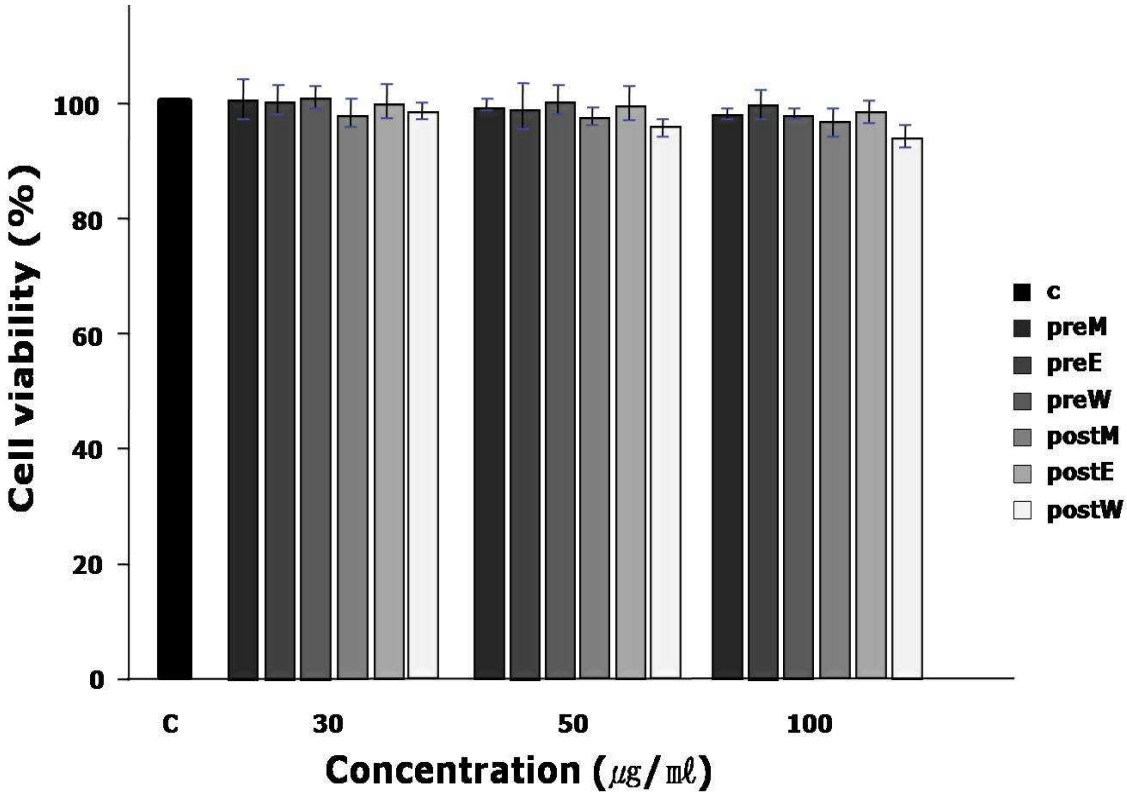

Fig. 7. Effects of CF extracts on cell viability of human hepatocellular carcinoma, SK-Hep-1. Cells were treated with the indicated concentrations of CF extracts for 24 hr. Levels of the cell viability were measured using the MTT assay. The viability of untreated control cells was defined as 100%. Results shown are means ±SD (n=3).

D. Effect of CF extracts on the activity of mushroom tyrosinase

Mushroom tyrosinase has been widely used as the target enzyme for screening and characterizing potential tyrosinase inhibitors. Because the mode of inhibition depends on the structures of both the substrate and inhibitors L-DOPA was used as the substrate in this study.

Table 3 showed the dose-dependent manner for the tyrosinase inhibitory effect of the CF extracts. It was found that all CF extracts had potent inhibitory effects on DOPA oxidase activity of mushroom tyrosinase and the inhibitory activities increased with increase of extract concentration. The results showed that postW had highest tyrosinase inhibitory activity among the extracts, followed by postM, postE, preE, preM and preW at 2 ㎎/㎖.

From a comparison of the other study, the tyrosinase inhibitiory activity of CF extracts was found to be significantly more pronounced. The published to tyrosinase inhibitory effect in the other *Graptopetalum paraguayense* E. Walther extracts (to obtain 75% tyrosinase inhibitory activity, the concentrations needed for ethanol 95%, ethanol 50%, water extracts were 1.76, 2.38 and 7.07 ㎎/㎖, respectively) (Keh-Feng et al., 2004) and *Quercus robur L.* Aerial parts, *Quercus robur* L. Bark, *Quiscualis indica* L. Aerial parts and *Salvia Africana lutea L.* Aerial parts were 25%, 20%, 39% and 48% at 500 ㎍/㎖, respectively (S. Momtaz et al., 2008).

Table 3. Tyrosinase inhibition activity of chestnut flower extracts.

Sample		Inhibition rate (%)		
		Concentration (mg/ml)		
		1	1.5	2
Prebloomed	Methanol extract	72.49%	76.06%	79.54%
	Ethanol extract	70.02%	77.19%	79.95%
	Water extract	64.14%	68.49%	71.08%
Postbloomed	Methanol extract	72.25%	83.42%	85.19%
	Ethanol extract	65.14%	76.14%	83.19%
	Water extract	63.38%	76.01%	89. 30%

E. Inhibition of melanin production in SK-MEL-2

Melanin synthesis in animals proceeds from tyrosine through a series of enzymatic and chemical steps initiated by tyrosine hydroxylation to yield L-DOPA, and subsequent L-DOPA oxidation to L-DOPA quinine. A single enzyme that catalyzes both reactions in tyrosinase (monophonol monooxygenase), a melanocyte-specific copper-containing glycoprotein located within specialized organelles called melanosomes (K. Jones et al., 2002). CF extracts did not show any toxicity on the melanocyte cells (SK-MEL-2) even at the highest concentration tested (100 ㎍/㎖, Fig. 8). PreM demonstrated 83.19% inhibition of melanin production at 100 ㎍/㎖ (Fig. 9).

Seventy percent ethanol extract of *M. alba* shows 7% and 10% inhibition of melanin production at concentrations 5 and 10 ㎍/㎖ respectively (Kim et al., 2006). Arbutin exhibited 2% inhibition of melanin production at 50 ㎍/㎖ and 100 ㎍/㎖, while more than 90% of murine melanocyte cells were viable. (Kim et al., 2006)

F. Reduction of tyrosinase mRNA and protein in SK-MEL-2 cells

To evaluate the effects of CF extracts on the expression of tyrosinase in SK-MEL-2 cells, CF extracts at a dose of 100 ㎍/㎖ were gavaged for 48 hr. Control group received no drug. Tyrosinase mRNA levels were determined by RT-PCR. Protein levels were measured by Western blot. As shown in Fig. 10 A

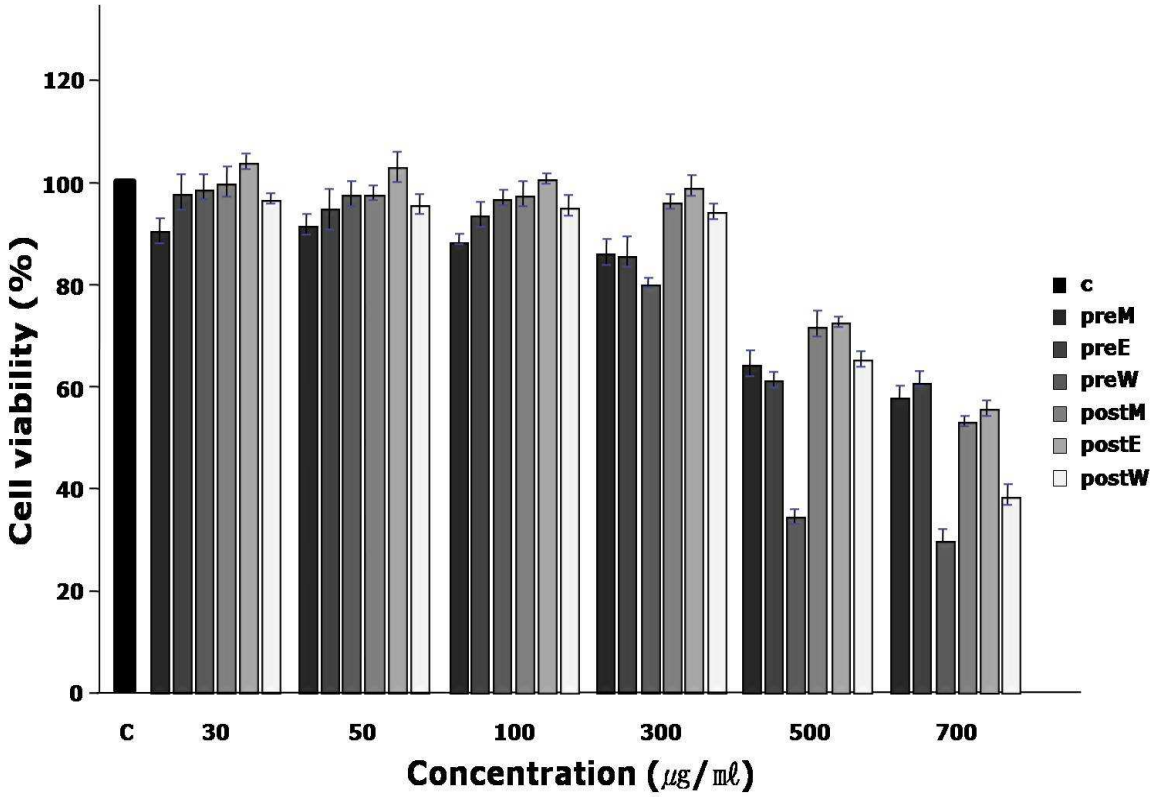

Fig. 8. Effects of CF extracts on cell viability of human melanoma, SK-MEL-2. Cells were treated with the indicated concentrations of CF extracts for 24 hr. Levels of the cell viability were measured using the MTT assay. The viability of untreated control cells was defined as 100%. Results shown are means ±SD (n=3).

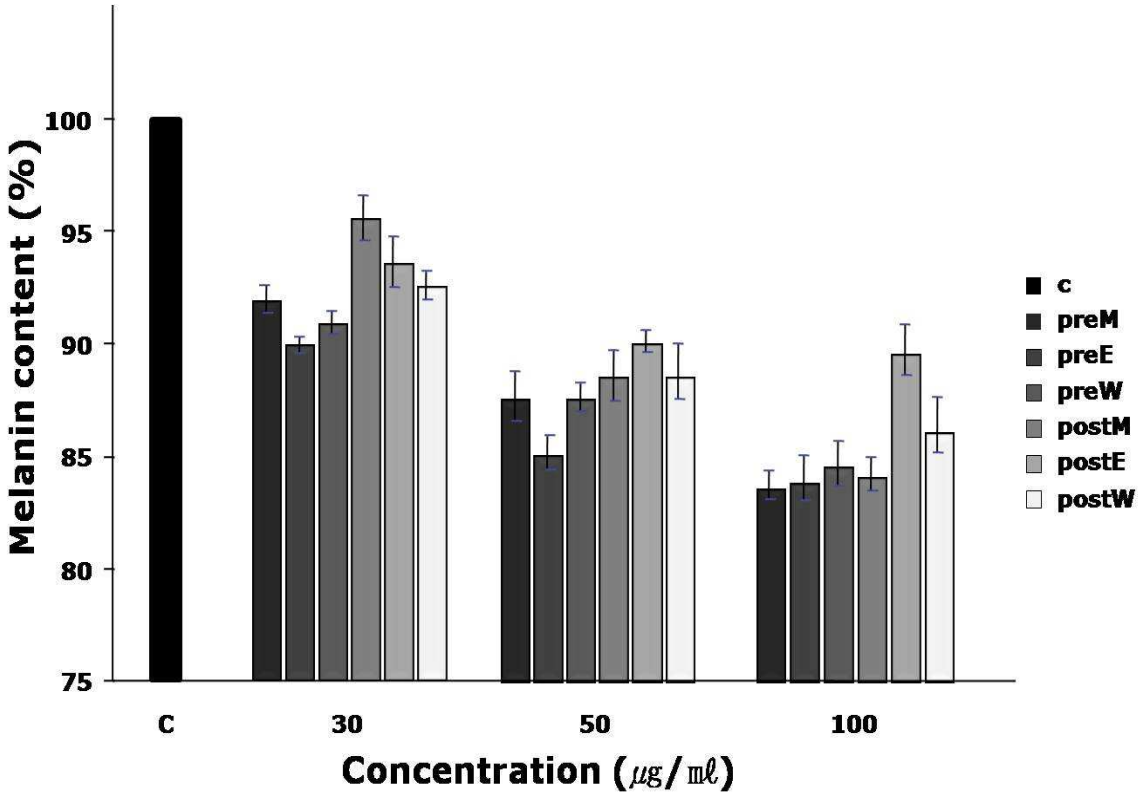

Fig. 9. Inhibitory effect of CF extracts on melanin synthesis in SK-MEL-2. Melanin content was measured at 405 ㎚. Results were expressed as % control.

and B, Tyrosinase was reduced by 100 ㎍/㎖ preM (40%), preE (17%), preW (22%) and postE (50%), but postM (8%) and postW (9%) were not reduced particularly. This results have also significantly inhibited the expression of prebloomed extracts treatment tyrosinase mRNA than prebloomed extracts treatment.

To determine whether CF extracts reduced expression of tyrosinase mRNA is accompanied by decrease in tyrosinase protein levels, western blot analysis was performed with anti-tyrosinase antibody. As shown in Fig. 11 A and B, tyrosinase protein levels were decreased over 50% at the dose of 100 ㎍/㎖ preM, preE, preW, postM and postE treatment. PostW was decreased over 10% at the dose of 100 ㎍/㎖. The protein levels expression of tyrosinase was consistent with the result from mRNA reduction (fig. 10 A and B).

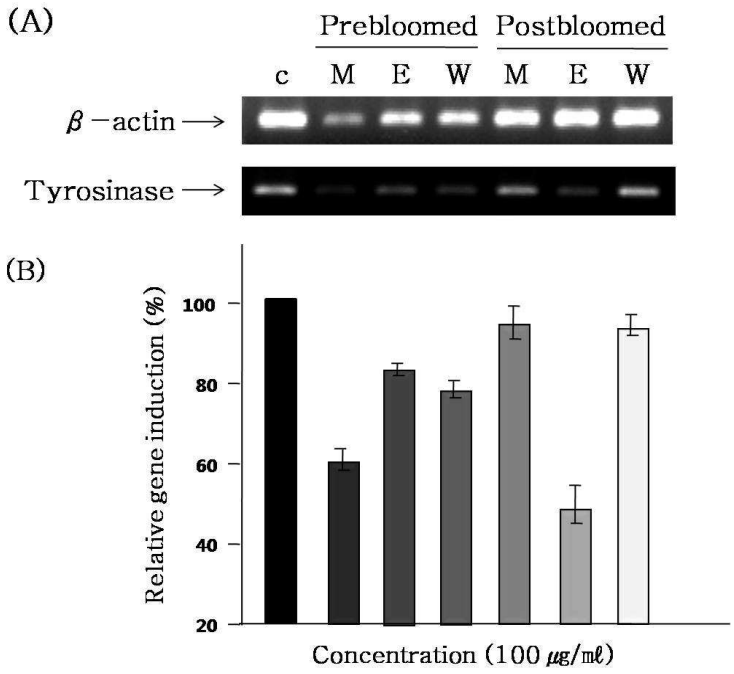

Fig. 10. Effect of different doses of CF extracts treatment on tyrosinase mRNA levels in SK-MEL-2 cells. Cells were treated with indicated concentrations of CF extracts as described under materials and methods. mRNA was analyzed by RT-PCR. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of mRNA in the non-treated control group taken as 100%. M: methanol extract, E: ethanol extract, W: water extract.

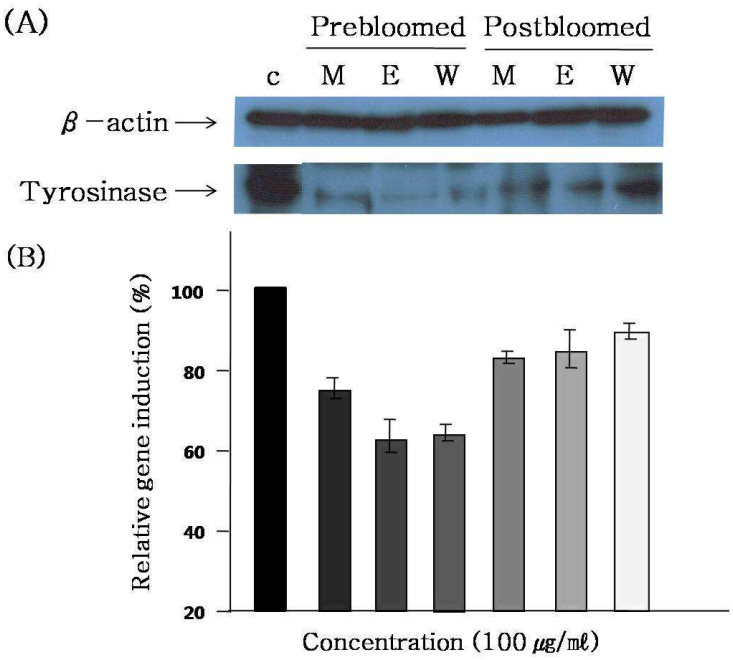

Fig. 11. Effect of CF extracts treatment on tyrosinase protein levels in SK-MEL-2 cells. Cells were treated with indicated concentrations of CF extracts as described under materials and methods. Protein was analyzed by western blot. Representative western blot analysis and quantification of relative intensity data are shown for tyrosinase protein levels. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of tyrosinase protein in the non-treated control group taken as 100%. M: methanol extract, E: ethanol extract, W: water extract.

IV. DISCUTION

Antioxidants are closely related to their biofunctionalities, such as the reduction of chronic disease like DNA damage, mutagenesis, carcinogenesis which is often associated with the termination of free radical propagation in biological systems (Zhu et al., 2002). Thus, antioxidant activity is widely used as a parameter for medicinal bioactive components. Phenolic and flavonoids had multiple pharmacological properties such as antioxidant, vasodilatory, anticarcinogenic, antiinflammatory, antibacterial, immune-stinulation, antiallergic and antiviral activities (Duarte et al., 1993; Gao et al., 1999; Wong and McLean, 1999).

In this study, the andtioxidant activity, cell viability and potential cosmeceutical effect of chestnut flower extracts were evaluated by scavenging activities of free radicals (DPPH), cell viability and inhibitory effect of tyrosinase.

The CF extracts were prepared in the investigation. This study showed that the methanol, ethanol and water extracts from chestnut flower had high total phenolic compound and total flavonoid compound. However, prebloomed extracts and postbloomed extracts shown a similar level of free radical scavenging activity (fig. 3), reducing power activity (fig. 4).

We have demonstrated that at the effective concentrations, CF extracts were not cytotoxic on the normal cell lines such as NIH3T3. The viability of cell were rather increased significantly with low concentrations of CF extracts (Fig. 5). On the contrary, CF extracts reduced cell proliferation and viability of Calu6 cells in

dose-dependent manners. This CF extracts mediated toxic effect was significantly more pronounced in human lung cancer cell lines than human hepatocellular carcinoma cells (Fig. 6 and 7). Recently, target organ specific agents that are capable of inducing selective apoptosis of cancer cells, but are harmless to normal cells are receiving considerable attention in the fields of cancer prevention and therapy (Mukherjee et al., 2001). Previously, various biological activities of Chestnut have been reported, most notably antioxidant activity and phenolic content (G. Va´ zquez, 2008 and Joa˜o C.M. Barreira, 2008), and antitumor activities (Yong-Soo Lee et al., 1999) and suggested that flavonoids and phenolic compounds were the major active compounds responsible for the biological activity of CF extracts.

Moreover, the present study demonstrates that CF extracts reduce expression of tyrosinase in a concentration-dependent manner. In addition, we detected consistent downregulation of tyrosinase mRNA and proteins by CF extracts. These results suggest that CF extracts have inhibitory effect on tyrosinase expression. Prebloomed extracts (methanol and ethanol extract) were shown to be potent tyrosinase inhibition activity and melanin synthesis inhibitors in SK-MEL-2. In addition to extracts of chestnut seed which are currently in use as cosmetic additives, results of this study indicate that extracts of prebloomed and postbloomed are likely to be useful for cosmetic applications and products. Their bio-guided isolated components may prove to have considerable value as cosmetics additives in the future.

Ⅴ. REFERENCES

An, B.J., Kwak, J.H., Park J.M., Lee, J.Y., Park, T.S., Son, J.H., Jo, C., Byun, M.W. Inhibition of enzyme activities and the antiwrinkle effect of polyphenol isolated from the persimmon leaf (*Diospyros kaki* folium) on human skin. *Dermatologic Surgery*. **31**: 848-854 (2005)

Arife A, Bengi E, Yelda C, Deniz A. Antioxidant activity and proline content of leaf extracts from *Dorystoechas hastate*. *Food Chemistry*. **111**: 400-407 (2008)

Bilodeau, M.L., Greulich, J.D., Hullinger, R.L., Bertolotto, C., Ballotti, R., Andrisani, O.M., BMP-2 stimulates tyrosinase gene expression and melanogenesis in differentiated melanocytes. *Pigment Cell Res*. **14**: 328-336 (2001)

Cai, Y., Luo, Q., Sun, M., & Corke, H. Antioxidant activity and phenloic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences*. **74**: 2157-2184 (2004)

Chen, J. S., Wei, C., Rolle, R. S., Otwell, W. S., Balaban, M. O., & Marshall, M. R. Inhibitory effect of kojic acid on some plant and crustacean polyphenol oxidases. *Journal of Agricultural and Food Chemistry*. **39**(8): 1396-1401

(1991)

Chomczynski P.A., Reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples, *BioTechniques*. **15**: 532-537 (1993)

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocher, P., & Vidal, N. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry*. **97**: 654-660 (2006)

Dooley, T. P. Topical skin depigmentation agents: Current products and discovery of novel inhibitors of melanogenesis. *Journal of Dermatological Treatment*. **8**(4): 275-279 (1997)

Dorman, H. J. D., Peltoketo, A., Hiltunen, R., & Tikkanen, M. J. Characterization of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. *Food Chemistry*. **83**: 255-262 (2003)

Dragsted, L. O., Strube, M., & Larsen, J. C. Cancer-protective factors in fruits and vegetables: biochemical and biological background. *Pharmacology and Toxicology*. **72**: 116-135 (1993)

Duarte J., Perez-Vicaino F., Zarzuelo A., Jimenez J., and Tamargo J., Vasodilator

effects of quercetin in isolated rat vascular smooth muscle. *European journal of pharmacology*. **239**: 1-7 (1993)

Gao Z., Huang K., Yang X., and Xu H., Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochimica et Biophysica Acta*. **1472**: 643-650 (1999)

Gutteridge, J. M. Free radicals in disease processes: A compilation of cause and consequence. *Free Radical Research*. **19**: 141-158 (1993)

G. Va´ zquez, E. Fontenla, J. Santos, M.S. Freire, J. Gonza´ lez-A´ lvarez*, G. Antorrena., Antioxidant activity and phenolic content of chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Industrial crops and products*. **28**: 279–285 (2008)

Halliwell, B. Antioxidants and human diseases: A general introduction. *Nutrition Review.* **55:** 44-52 (1997)

Halliwell, B., Gutteridge, J. M., & Cross, C. E. Free radicals, antioxidants, and human disease: where are we now. *Journal of Laboratory and Clinical Medicine*. **119**: 598-620 (1992)

Hearing, V.J., Tsukamoto, K., Enzymatic control of pigmentation in mammals.

FASEB J. **5**: 2902-2909 (1991)

Hirokawa, S. Tennen Yakujishyo. Hirokawa Shyoten, Tokyo, Japan (1986)

Hua W, Xiang DG, Gao CZ, Lei C and Wen By. In vitro and in vivo antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit. *Food Chemistry*. **106**: 888-895 (2008)

Ji-Young Kim, Jung-A Lee, Kil-Nam Kim, Weon-Jong Yoon, Wook-Jae Lee and Soo-Yeong Park. Antioxidative and Antimicrobial Activities of *Sargassum muticum* Extracts. *J Korean Soc Food Sci Nutr*. **36**(6): 663-669 (2007)

K. Jones, J. Hughes, M. Hong, Q. Jia, S. Orndorff. Modulation of Melanogenesis by Aloesin: A Competitive Inhibitor of Tyrosinase. *Pigment Cell Research*. **15**: 335-340 (2002)

Kahn, V., Ben-Shalom, N., & Zakin, V. (1997). Effect of kojic acid on the oxidation of N-acetyldopamine by mushroom tyrosinase. *Journal of Agricultural and Food Chemistry*. **45**(11): 4460-4465 (1997)

Keh-Feng Huang, Yi-Wei Chen, Chen-Tien Chang, Su-Tze Chou. Studies on the inhibitory effect of *Graptopetalum paraguayense* E. Walther extracts on

mushroom tyrosinase. *Food Chemistry*. **89**: 583-587 (2005)

Kim, M., Meng, X., Kim, A., Wang, M., Simon, J.E. Developing a longlasting tyrosinase inhibitor from *Morus alba* L. *Cosmetics & Toiletries Magazine*. **121**: 91-102 (2006)

Knight, J. A. Diseases related to oxygen-derived free radicals. *Annals of Clinical and Laboratory Sciences*. **25**: 111-121 (1995)

Kubo, I., & Kinst-Hori, I. Flavonols from saffron flower: Tyrosinase inhibitory activity and inhibition mechanism. *Journal of Agricultural and Food Chemistry*. **47**(10): 4121-4125 (1999)

Kubo, I., & Kinst-Hori, I. Tyrosinase inhibitors from cumin. *Journal of Agricultural and Food Chemistry*. **46**(12): 5338-5341 (1988)

Lee YL, Yang JH and Mau JL. Antioxidant properties of water extracts from *Monascus fermented* soybeans. *Food Chemistry*. **106**: 1128-1137 (2008)

Liu, R. H. Health benefits of fruits and vegetables are from additive and synergistic combination of phytochemicals. *American Journal of Clinical Nutrition*. **78**: 517S-520S (2003)

Maeda, K., & Fukuda, M. In vitro effectiveness of several whitening cosmetic components in human melanocytes. *Journal of the Society of Cosmetic Chemists*. **42**: 361-368 (1991)

Maertinez, M.J., Betancourt, J., Alonso-Gonzalez, N., Jauregui, A. Screening of some Cuban medicinal plants for antimicrobial activity. *J. Ethnopharmacol*. **52**: 171-174 (1996)

Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpam, N., Neungton, N., Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell line. *J. Ethnopharmacol* **90**: 161-166 (2004)

Mosher, D. B., Pathak, M. A., & Fitzpatrick, T. B. Vitiligo: Ethology, pathogenesis, diagnosis and treatment. In T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg, & K. F. Austen (Eds.), Updates: *Dermatology in general medicine*. New York: Mcgraw-Hill. 373-398 (1983)

Mukherjee A.K., Basu S., Sarkar N., and Ghosh A.C., Advances in cancer therapy with plantbased natural products, *Current medicinal chemistry*. **8**: 1467-1486 (2001)

N. Ozsoy, A. Can, R. Yanardag, N. Akev. Antioxidant activity of *Smilax excelsa* L.

leaf extracts. *Food Chemistry*. **110**: 571-583 (2008)

S. Momtaz, N. Lall, A. Basson. Inhibitory activities of mushroom tyrosinase and DOPA oxidation by plant extracts. *South African Journal of Botany*. **74**: 577-582 (2008)

Seo, S. Y., Sharma, V. K., & Sharma, N. Mushroom tyrosinase: Recent prospects. *Journal of Agricultural and Food Chemistry*. **51**(10): 2837-2853 (2003)

Shin, N. H., Ryu, S. Y., Choi, E. J., Kang, S. H., Chang, I. M., Min, K. R., & Kim, Y. Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. *Biochemical and Biophysical Research Communication*. **243**(3): 801-803 (1998)

Suresh KK, Ganesan K and Subba PVR. Antioxidant potential of solvent extracts of *Kappaphycus alvarezii* (Doty) An edible seaweed. *Food Chemistry*. **107**: 289-295 (2008)

Vita, J. A. Polyphenols and cardiovascular diease: effects on endhothelial and platelet function. *American Journal of Clinical Nutrition*. **81**: 292S-297S (2005)

Wong W.S., and McLean, A.E., Effects of phenolic antioxidants and flavonoids on

DNA synthesis in rat liver, spleen, and testis *in vitro*. *Tosicology*, **139**: 234-253 (1999)

Yang, L.L., Lee, C.Y., Yen, K.Y. Induction of apoptosis by hydrolysable tannins from *Eugenia jambos* L. on human leukaemia cells. *Cancer Lett*. **157**: 65-75 (2000)

Yanga, J.-H., Linb, H.-C., Maub, J.L. Antioxidant properties of several commercial mushrooms. *Food Chemistry*. **77**: 229-235 (2002)

Yong-Soo Lee, Kwon-Il Seo, and Ki Hwan Shim, Antimicrobial activities of chestnut flower extracts (*Castanea crenata*). *Korean J. POSTHARVEST SCI. TECHNOL*. **6**: 104-109 (1999)

Zhou, K., Laux, J. J., Yu, L. Comparison of Swiss red wheat grain and fractions for their antioxidant properties. *J. Agric. Food Chem*. **52**: 1118-1123 (2004)

Zhou, K., Yu, L. Total phenolic contents and antioxidant properties of commonly consumed vegetables grown in Colorado. *Lebensmittel-Wissenschaft und-Technologie*, **39**: 1155-1162 (2006)