New Antioxidant Sources; Tinged Autumnal Leaves of Maple and Cherry Trees

Jeong-Jae Lee, Chung-Woo Lee, Young-Ho Cho, Sung-Min Park,
Bum-Chun Lee and Hyeong-Bae Pyo
Hanbul Cosmetics Co., R & D center, Chungbuk, Korea

Abstract:

Living systems constantly suffer from atmospheric reactive oxygen species and also produce inevitably them by the course of metabolism. Therefore, antioxidants play important roles in protecting the systems from various diseases caused by them. In this study, we investigated various tinged autumnal leaves as antioxidant sources. Among them, the red leaf extracts of *Acer Palmatum THUNBERG.*(*Aceraceae*: maple tree) and *Prunus Donarium Sieb. Var. spontanea Makino*(*Rosaceae* : cherry tree) showed the highest anti-oxidativities. The major antioxidants in their red leaves were isolated and identified as vitexin from maple leaves and isoscutellarein-4'-O-β-glucopyranoside from cherry leaves. Finally, we evaluated the efficacy of skin care products containing the extracts by human use study. As a result, the tinged leaves of maple and cherry trees were evaluated as good antioxidant sources on the bases of antioxidativities, cytotoxicities, cell proliferation effects and human use study.

Introduction

Reactive oxygen species (ROSs) is belived to cause various diseases by oxidative stress to biological systems, especially lipids within cell membrane. Human body continually produces inevitable ROSs (such as hydroxyl radical, superoxide, and other singlet oxygens), called intrinsic ROSs, because biological systems require them in metabolic redox systems. Moreover, we are surrounded with extrinsic ROSs derived from the excess exposure to UV-ray and air pollution. Lipid peroxidation initiated by these ROSs is believed to damage cells directly or indirectly by the loss of those polyunsaturated fatty acids of cell membranes.[1-3] The produced lipid peroxides and their secondary products such as reactive carbonyl compounds may damage cellular constituents.[1,4-6] Therefore, excess production and inflow of ROSs within tissues may ultimatley cause various diseases such as cancer and other disorders by damage of DNA, lipids, proteins and carbohydrates in cells. Which is the most important target of damage depends upon the cell type subjected to the oxidative stress and how it is imposed.[7]

Living systems including human body have their intrinsic oxidation-protecting systems such as superoxide dismutase(SOD), catalase, and glutathione peroxidase. But the systems may be insufficient to protect themselves from excessively inflowing ROSs. Therefore, intake of food-derived substances such as tocopherols, ascorbic acid, carotenoids and flavonoids is required to diminish the undesired effects caused by oxidation processes in organism[1]. Unfortunately, it is difficult to regulate easily these excess ROSs production. Therefore, many research groups have searched for more effective antioxidants and effective application methods to regulate ROS equilibrium in body. Recently, natural antioxidants from

plants have been received attention. In particular, flavonoids and polyphenol compounds, which are widely distributed in the plant kingdom, show remarkable promise for a wide range of pharmacological use for allergies, inflammation, antiviral, antitumor and diabetes.[8-10] And the active derivatives have been synthesized on based on the study of the correlation of structure and activity.

In this study, we focused on the possibility of tinged autumnal leaves as antioxidant sources. The seasonal changes vary growing process of plants and temperature and sunshine duration are major factors to affect the color change of leaves. In fall, green leaves gradually turn red or yellow because red and yellow colors originate from xanthophyll, carotenoids and flavonoids which appear with the destruction of chlorophyll. So they have secondary metabolites at a high rate compared to green leaves, which contain abundantly primary metabolites. Therefore, the autumnal leaves were expected to show the increase of desired activity. First we screened various tinged autumnal leaves to select target materials with potent antioxidant activities and identified the major active compound contained in them.

In this report, we describe the possibility of tinged autumnal leaves as antioxidants, their active substances and applications to cosmetics.

Methods and Materials

Melting points were not corrected. IR spectra were obtained with Jasco FT-IR 5300 and UV-VIS spectra were obtained with Varian carry IE spectrophotometer. ^{1}H-NMR(300 MHz) and ^{13}C-NMR (75MHz) spectra were obtained with Bruker 300 MHz NMR spectrometer.

1. Preparation of Extracts

The tinged autumnal leaves of maple tree (*Acer Palmatum THUNBERG.* (*Aceraceae*))[11] and cherry tree (*Prunus Donarium Sieb. Var. spontanea Makino*(*Rosaceae*))[12] were selected as final antioxidant materials by screening works. The leaves were collected between October and November in the middle part of South Korea. The 100g of each leaf was extracted with ethanol for 10 days at room temperature and each ethanol extract was concentrated *in vacuo*. And then 40 % aqueous 1,3-butylene glycol (1,3-BG) extracts were prepared by the same method for the applications to cosmetics.

2. Antioxidative Activities of the Extracts

The ethanol and 40% 1,3-BG aqueous extracts were diluted to 3 % with ethanol to prepare samples. 3% ethanol solutions of *dl*-α-tocopherol (Aldrich co.), 3% aqueous solution of green tea extracts (Bioland co., Korea) and 3 % aqueous solution of *l*-ascorbic acid (Sigma-Aldrich co.) were prepared as comparative standards. The antioxidative activity of each sample was evaluated by nitroblue tetrazolium chloride mono hydrate (NBT; Acros organics) test and 1,1-diphenyl-2-picrylhydrazyl(DPPH; Waco pure chemical co.) test with suitable dilution as follows. In NBT test, 0.1mL sample, 2.4mL Na_2CO_3 buffer(0.05M, pH

10.2), 0.1mL xanthine (3mM, Sigma co.), 0.1mL ethylene diamine tetraacetic acid (EDTA; 3mM; Sigma co.), 0.1mL NBT solution(0.75mM) and 0.1mL bovine serum albumin(BSA; Sigma co.) solution were added in turn to each test tube and reacted at 25℃ for 20 min. To each test tube, 0.1mL xanthine oxidase(Sigma co.) was added and reacted at 25℃ for 20 min. The reaction was quenched with 0.1mL $CuCl_2$ (6mM; Sigma co.). Finally inhibitory effect (%) was calculated as compared with blank control after measuring absorbance at 560nm. In DPPH test, 0.1ml of the above sample and 3.9ml of DPPH alcoholic solution (0.06mM) was mixed in a tube for 30 seconds and incubated for 30 minutes at room temperature. And the absorbance was measured at 515nm. Then, its antioxidative activity (%) was calculated with respect to blank control.

3. Cytotoxicities and Cell Proliferation Effects of the Extracts

Human fibroblast (ATCC, Hs68), which was cultured in Dulbecco's Modified Eagle's Medium(DMEM; GibcoBRL) supplemented with 10% fetal bovine serum (FBS; TerraCell international S.A.) under atmosphere of 5% CO_2 and humidity of 100% at 37℃, was used to assay the cytotoxicities of tinged autumnal leaf extracts. $1x10^6$ cells/mL were seeded into a 96-well microplate and incubated at 37℃ for 24 h. Samples sterilized with 0.2 micrometer filter were added at various concentrations to determine LD_{50} values and incubated at 37℃ for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent(Sigma co.) was added and incubated at 37℃ for 4 h. After removing culture medium, 1N NaOH *iso*-propanol solution was added and stirred for 20min and then absorbance was measured at 565nm. The cytotoxicities of the extracts were determined as compared with non-sample treated controls by observing viability of them. The criterion of evaluation was the LD_{50} value of each sample.

To evaluate the cell proliferation effects of the extracts, we first screened the maximum concentration to avoid cell death. The same cells as above were used to assay the cell proliferation effects. 2×10^4 cells/mL were seeded into a 96-well microplate and incubated at 37℃ for 24 h. After replacing the medium with 0.5% FBS DMEM, samples at a concentration to avoid cell death were applied and incubated at 37℃ for 48 h. They were stirred for 20min after bicinchoninic acid (BCA; Pierce) solution was added and incubated at 37℃ for 30min. Finally, the cell proliferation effects (%) of tinged autumnal leaf extracts were determined by comparing with negative and positive controls after measuring absorbance at 565nm.

4. Emulsion containing the Extracts:

To test emulsion types compatible with these extracts, several types of emulsions (o/w, w/o, w/s and multi-phase emulsions) were prepared. To examine thermal stability for 6 weeks, each emulsion was incubated in 4, 25, 42℃ incubators and variable circulator which is able to vary temperature from -10℃ to 42℃ every 3 days. Both UV and fluorescent radiation were applied to each emulsion for 6 weeks to

observe the behavior of each emulsion on light. Finally, we selected an optimized type of emulsion from all the data acquired.

5. Anti-Wrinkle Effect of Emulsion containing theExtracts

To determine the effect of selected emulsion sample on cutaneous relief on human subjects, we requested human use study to Laboratoire DERMSCAN(Lyon/Villeurbanne, France). We considered an open and intra-individual study. Volunteers consisted of 7 healthy caucasian females, aged between 43 and 51(47± 1), were selected strictly by study criteria. The volunteers also had wrinkles around eye area (crow's feet). The sample was applied to the crow's feet zone twice daily at home for 56 days. The experimental conditions during measurements were maintained constantly under the ambient temperature of 24± 2℃ and relative humidity between 40-60 %. The treated zone was compared to the non-treated zone after twice-daily use for two months. Quantitative measurements of the effect on cutaneous relief were done using the Skin Image Analyzer®(Monaderme co.). Oblique lighting (35°) brings shadows from the replica to the fore, which are then observed with CCD camera linked to a computer. An area of 1 cm^2 is studied. It produces a digitized image enabling a roughness index to be obtained by analyzing the shades of gray. This roughness index characterizes the skin surface relief.[13,14] Studied parameters include the total wrinkled surface, the number and average depth of cutaneous microrelief furrows, median wrinkles and deep wrinkles using Quantrides® software (Monaderme co.). Microrelief furrows have a depth inferior to 55 micrometers. Median wrinkle furrows have a depth between 55 and 110 micrometers and deep wrinkle furrows have a depth longer than 110 micrometers. All furrows with a minimum surface of 0.03 mm^2 are detected. Finally, the data gathered were treated to judge the efficacy of sample by study criteria.

6. Isolation and Identification of Major Active Substances in Maple and Cherry Tree Leaves

Ethanol extracts of the leaves were roughly separated using solvent fractionation from the concentrates. First, non-polar mixtures in the concentrates were extracted with hexane and chloroform. The polar residues were separated with ethyl acetate and *n*-butanol in turn. A yellow precipitate obtained from ethyl acetate fraction of maple tree leaf extract was filtered and recrystallized from methanol. We named the compound antioxidant A (110mg, 0.11%). *n*-Butanol fraction of cherry tree leaf extract, which possessed the potent activity, was briefly examined to confirm the constitution, chemical and structural properties of constituents using a series of visualizing reagents such as $FeCl_3$ (Sigma-Aldrich co.), Chloramin T (Showa chemical co.) and N-bromosuccinimide (NBS; Aldrich co.). The fraction was separated into various subfractions on silica gel column chromatography (230-400mesh, Silica gel $60F_{254}$, Merck) using chloroform-ethyl acetate-methanol (1:1:1) as an eluent. After examined by NBT test, the subfraction which had the potent activity was concentrated and further purified by flash column chromatography. The compound was named antioxidant B (70mg, 0.07%). The chemical structures of antioxidant A and B

were identified using FT-IR, ^{1}H, ^{13}C-NMR (DMSO-d_6) and UV-VIS spectra.

The antioxidative activities of antioxidant A and B were compared with other well-known antioxidants such as flavonoids and vitamin E by NBT test.

RESULTS AND DISCUSSION

1. Antioxidative Activities of the Extracts

The activities of extracts were as good as or higher than green tea extracts but not as high as vitamin E as shown in figure 1 representing the results of NBT and DPPH tests on the extracts compared with well-known antioxidants. Antioxidativity of vitamin C couldn't be evaluated by NBT test. Maybe vitamin C doesn't react superoxide ion produced from reaction between xanthine and xanthine oxidase but directly with nitroblue tetrazolium. In DPPH test, all the samples showed very high activities. However, these samples didn't have the antioxidativities as high as vitamin E and C. The ethanolic extracts showed typically more potent effects than 1,3-BG extracts as illustrated in figure 1. These results are due to ethanol being the most suitable solvent to extract active constituents from plants. But ethanol can't be used directly in cosmetics because of the destruction of emulsion and mild toxicity, while the 1,3-BG have been generally used as an excellent extracting solvent as well as a humectant in cosmetics.

2. Cytotoxicities and Cell Proliferation Effects of the Extracts

A series of 1,3-BG extracts showed generally far lower cytotoxicities than ethanol extracts(figure 2). The LD_{50} values of 1,3-BG maple and cherry leaf extract were 2.4% and 1.6% respectively, while those of ethanol extracts were 0.64%. And 1,3-BG cherry leaf extract showed considerably higher proliferation effect than the other samples. And ethanol extracts showed low effects on the cell because of those low LD_{50}. On the other hand, 1,3-BG maple leaf extract didn't show effect remarkably. From these results, two types of 1,3-BG extracts were evaluated to be capable of being applied safely to skin care products within 5-10 %(wt/wt) without any problem.

3. Emulsion containing the Extracts

These extracts showed broad compatibility with all types of emulsions without the disruption or degeneration under harsh conditions. But w/o type was thought to have a problem for daily use because of oiliness and thickness. W/s type was not suitable for using for long term because of giving dry feeling to skin immediately after applied to face. In case of multi-phase emulsion, severe instabilities on thermal and other physical shocks were observed. As a result, we selected o/w type emulsion (formula 1), giving a fresh, moisturizing effect. Users could make use of the sample without any inconvenience.

40% 1,3-butylene glycol extract of tinged autumnal leaf of maple tree	5.00%
40% 1,3-butylene glycol extract of tinged autumnal leaf of cherry tree	5.00
Cetanol	2.50
Mineral oil	10.00
Soybean oil	5.00
Dimethicone	2.00
1,3-Butylene glycol	5.00
Glyceryl stearate	2.00
PEG20 Methylglucose sesquistearate	1.00
Sorbitan monooleate	0.30
Carbomer(2% aqua)	5.00
Preservatives	qs
Water(aqua)	qs 100.00

Formula 1. *O/w Emulsion containing Tinged Autumnal Leaf Extracts*

4. Anti-Wrinkle Effect of Emulsion containing theExtracts

In this study, no allergic or irritation reaction was observed. Figure 3 represents the results about the percentage of volunteers showing effects. Figure 4 shows the quantitative results on cutaneous wrinkle relief effect of emulsion containing the extracts in each measured item. The results, however, are only first tendencies due to the few number of volunteers (n=7). After 56 days, the sample showed a decrease in the number of deep wrinkles for five volunteers out of the seven (71%). Moreover the number of average wrinkles and the total wrinkled surface decreased for four volunteers (57 and 57% respectively). Quantitative results with the confidence interval at 95% showed the decrease of the number and depths of wrinkles as well as total wrinkled surface. From these results, tinged autumnal leaf extracts are thought to be considerably effective on wrinkle-relief.

5. Major Active Substance in Maple and Cherry Leaves

Antioxidant A obtained from ethyl acetate fraction of maple leaves showed a dark brown color in $FeCl_3$ test and yellow color in chloramin T test. Its R_f value on TLC was 0.74 ($CHCl_3$-ethyl acetate-EtOH=1:1:1). From these results, antioxidant A was estimated as a flavonoid and its detailed chemical structure was identified as vitexin (apigenin-8-*C*-β-D-glucopyranoside) and elucidated comparing with reported data[13-17] (figure 6). The spectrum data and physicochemical properties of antioxidant A are as follows.

Mp 250~252℃, IR (KBr): 3383, 1655 (α,β-unsaturated carbonyl), 1614, 1508, 1429 (aromatic double bond) cm^{-1}, ^{1}H-NMR (DMSO-d_6): 8.13 (d, J=8.32Hz, H-2', 6'), 7.02 (d, J=8.32Hz, H-3', 5'), 6.73 (s, H-3), 6.39 (s, H-6), 4.91 (d, J=9.7Hz, β-anomer proton), 3.4-4.0 (m, D-glucose's protons) ppm. ^{13}C-NMR (DMSO-d_6): 183.1 (α,β-unsaturated carbonyl), 165.0 (C-2), 163.7 (C-7), 162.0 (C-4'), 161.5 (C-5), 157.1 (C-9), 129.5 (C-2', C-6'), 122.7 (C-1'), 116.6 (C-3', C-5'), 105.4 (C-8), 105.0 (C-10), 103.1 (C-3), 99.0 (C-6), 82.6 (C-3"), 79.9 (C-3"), 74.4 (C-1"), 71.9 (C-2"), 71.6 (C-4"), 62.4 (C-6"), 74.4 ppm. UV-VIS (methanol): λ_{max} 270, 336 nm.

Antioxidant B obtained from *n*-butanol fraction of cherry tree leaves showed a strong brown color in $FeCl_3$ test and brown color in chloramin T test. And its R_f value on TLC was 0.66 ($CHCl_3$-ethyl acetate-EtOH=1:1:1). From these results, antioxidant B was estimated as a flavonoid and its detailed chemical structure was identified as isoscutellarein-4'-O-β-glucopyranoside (ISTR-O-Glu) (figure 6). The spectrum data and physicochemical properties of antioxidant B are as follows.

Mp 163~166℃, IR (KBr): 3383, 1657 (α, β-unsaturated carbonyl), 1610, 1493, 1444 (aromatic double bond) cm^{-1}, ^{1}H-NMR (DMSO-d_6): 7.93 (d, J=8.85Hz, H-2', 6'), 6.99 (d, J=8.85Hz, H-3', 5'), 6.66 (s, H-3), 6.55 (s, H-6), 4.91 (d, J=9.78Hz, β-anomer proton), 3.4-3.9 (m, D-glucose's protons) ppm. ^{13}C-NMR (DMSO-d_6): 183.1 (α, β -unsaturated carbonyl), 165.1 (C-2), 164.4 (C-7), 162.5 (C-4'), 161.3 (C-5), 158.4 (C-8), 158.3 (C-9) 129.4 (C-2', C-6'), 122.5 (C-1'), 116.9 (C-3', C-5'), 109.4 (C-6), 105.0 (C-10), 103.3 (C-3), 95.6 (C-1''), 81.7 (C-3''), 80.0 (C-5''), 75.0 (C-2''), 71.1 (C-4''), 61.7 (C-6'') ppm. UV-VIS (methanol): λ_{max} 272, 337 nm.

The antioxidative activities of 0.3% ethanol samples of two flavonoids separated from the ethanolic extracts were compared with the 0.3 % ethanol solutions of reagent-grade flavonoids and vitamin E by NBT test. As shown on figure 5, ISTR-O-Glu and vitexin showed the antioxidative activities of about 90%. These results indicate that two types of flavonoids have antioxidative activities as good as vitamin E and other flavonoids known to have potent antioxidative activities.

CONCLUSON

We investigated various green and tinged autumnal leaves as new antioxidant sources for cosmetics. The crude tinged leaf extracts showed somewhat higher antioxidativities than green leaf extracts in general, especially the extracts of *Acer Palmatum THUNBERG.*(*Aceraceae*: maple tree) and *Prunus Donarium Sieb. Var. spontanea Makino*(*Rosaceae* : cherry tree). We also identified vitexin, a C-glycosyl flavonoid, from *maple tree leaves* and isoscutellarein-4'-O-β-glucopyranoside (ISTR-O-Glu) from cherry tree leaves respectively as the major antioxidants. But, other active compounds such as various alkaloids and other glycosyl flavonoids were discovered in the tinged autumnal leaf extracts in great quantities. So the potent antioxidativities of the extracts result from the synergic effect caused by these various compounds contained in the extracts. Finally, the emulsions containing these extracts were prepared and studied for physical properties. The emulsion was proven to be effective on cutaneous relief through human use study. Many researchers search for novel antioxidants and regulating methods against ROSs. But yet, it may be difficult to develop such antioxidants and methods for cosmetics. However, such a endeavor is desirable to go ahead continually for future.

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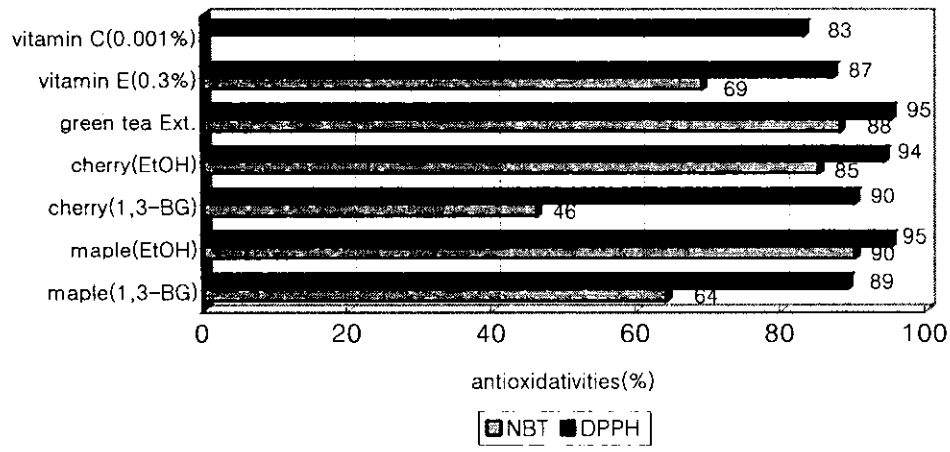


Figure 1. *Results of NBT and DPPH test on extracts*

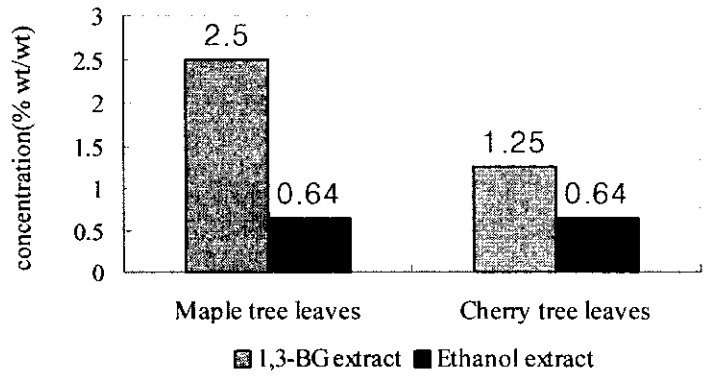


Figure 2. *LD_{50} values (% concentration) of extracts*

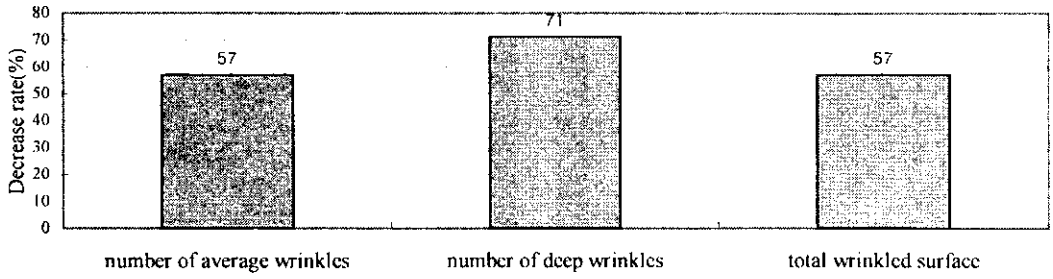


Figure 3. *Anti-wrinkle effect of emulsion containing th extracts*

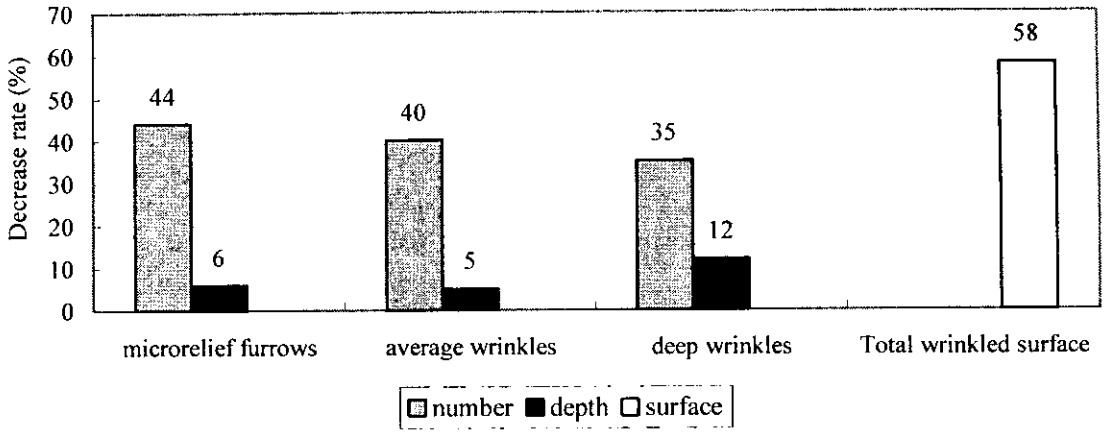


Figure 4. *Quantitative results on cutaneous wrinkle relief effect of emulsion containing the extracts*

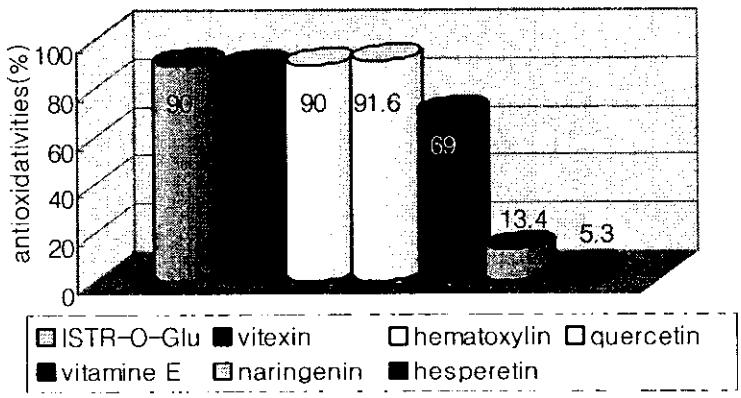
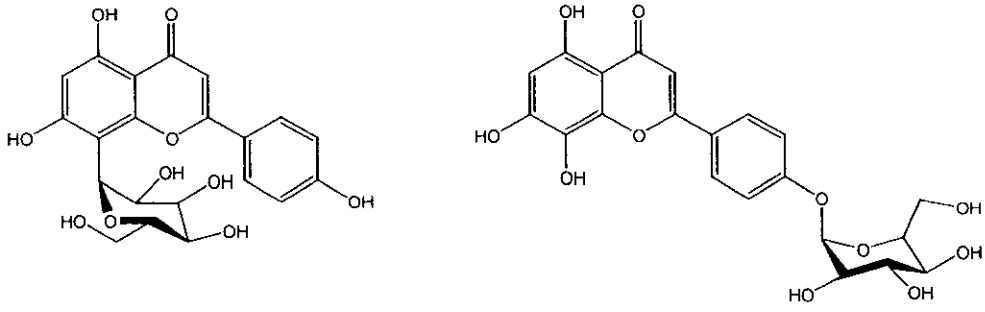


Figure 5. Results of NBT test on various substances



Vitexin (Antioxidant A)
(Apigenin-8-C-β-D-glucopyranoside)

ISTR-O-Glu (Antioxidant B)
(Isoscutellarcin-4'-O-β-D-glucopyranoside)

Figure 6. *Vitexin and ISTR-O-Glu (isoscutellarein-4'-O-beta-D-glucopyranoside)*