Inhibitory effects of the medicinal plant extracts on tyrocinase and elastase, and free radical scavenging effects.

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Abstract

One of the important function of skin is the protection from harmful environments. Many studies were carried out to keep the skin from wrinkling and pigmenting. Skin wrinkle and pigmentation could be caused by the unusual disruption of connective tissue, the formation of free radicals and the radiation of ultraviolet.

In this study, the extracts obtained from 23 different kinds of medicinal plants were examined whether they have inhibitory effect on tyrosinase and elastase as well as free radical scavenging effect. All the extracts examined were obtained by using 70% (v/v) ethanol at 60°C. It has been found that there were two medicinal plants which have positive effects matching with the purpose of this study.

The extract of *Ephedra sinica stapf* shows an inhibitory effect on tyrosinase (IC_{50} = 83.7μg/ml), on elastase (IC_{50} = 690μg/ml), and also shows free radical scavenging effect (IC_{50} = 29.6μg/ml).

The extract *of Betula platyphylla Var.* shows an inhibitory effect on elastase (IC_{50} = 498.1μg/ml), and free radical scavenging effect (IC_{50} =

9μg/ml).

The aim of the study is to find the natural compounds which have function in skin-lightening, anti-wrinkling and anti-oxidation, with safe and active ingredients in cosmetic.

Material and methods

Chemicals

1,1-dipheny-2-picrylhydrazyl (DPPH), Tyrosinase, Tyrosine, Elastase, Dimethyl sulfoxide (DMSO) and N-succinyl-ALA-AlA-AlA-p-nitroanilide (ESIV) were purchased from Sigma Chemical Co. (USA). Other chemicals and solvents were used the in analytical grade. High glucose dulbecco's modified elagle's medium (DMEM), antibiotics (streptomycin and Penicillin-G) and fetal bovine serum (FBS) were purchased from Gibco (USA).

Preparation of medicinal plant extract

All plants, used in this study, were shredded and powdered. The powder samples were extracted by using 70% (v/v) ethanol at 60°C for 3hr. After the sample was filtered through two layers of cheese-gause, the filter cakes were subjected to repeat the same procedure three times to increase the extraction yield. The initial extracts described above were mixed together and then filtered using a sheet of Whatman No. 1 filter paper. The filtrate was concentrated using an evaporator at 60°C.

Assay for an inhibitory effect on tyrosinase

An inhibitory effect on tyrosinase was examined whether each plant extract has an inhibitory effect on tyrosinase by measuring the change of the enzyme activity in a 96 well reader (Model; spectra MAX 340). The reaction was carried out in 50mM sodium phosphate buffer (pH 6.7) containing 1.5mM L-tyrosine and 40unit/ml mushroom tyrosinase. Each plant extract was added to the reaction mixture to a final concentration of 100 and 1000 μg/ml and examined the inhibitory effect on tyrosinase at 37°C after pre-incubation for 40min. The change of the absorbance at 475nm was measured using a 96 well reader. The percentage of inhibitory effect on tyrosinase was calculated as follows:

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

Where A is absorbance at 475nm without the plant extracts after incubation, and B is the change of the absorbance at 475nm with the plant extracts after incubation.

Assay for an inhibitory effect on elastase

The activity of porcine pancreatic elastase (PPE; Sigmachem. Co., Type IV) was examined using N-Succ-(Ala)3-nitroanilide as substrate, and measuring the release of p-nitroaniline at 410nm. The reaction was carried out in 100mM Tris-HCl buffer (pH 8.0) containing 0.29mM N-Succ-(Ala)3-nitroanilide and 0.104unit/ml elastase. Each plant extract was added to the reaction mixture to a final concentration of 100 and 1000 μg/ml and examined inhibitory effect on elastease at 25°C after pre-incubation for 60min.

The change of the absorbance was measured at 410nm by using a 96 well reader. The percentage of inhibitory effect on elastase was calculated as follows:

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

Where A is the change of the absorbance at 410nm without the plant extracts after incubation, and B is the change of the absorbance at 410nm with the plant extracts after incubation.

Assay for a scavenging effect on DPPH radical

The reaction was carried out in 100% ethanol containing 60μM DPPH and the extracts in which 66 and 660μg/ml of plant extract were employed. The scavenging effect against DPPH radical was at room temperature for 30min. The change of the absorbance was measured at 517nm by using a 96 well reader. The percentage of scavenging effect on DPPH radical was calculated as follows:

$$\text{Scavenging effect on DPPH radical}(\%) = [(A-B)/A] \times 100$$

Where A is the change of the absorbance at 517nm without the plant extracts after incubation, and B is the change of the absorbance at 517nm with the plant extracts after incubation.

Assay for the Cytotoxicity on the fibroblast cell line,NIH3T3.

Each extract from *Betula platylla Var.* and *Ephedra sinica Stapf* were dissolved using DMSO and then diluted to DMEM medium. These were added to each culture medium to a final concentration of 10, 30, 50, 100, 150 and 200 μg/ml. The cells were cultured at 37°C with 5% CO_2 for 48 hours. The inhibition of the cell growth was measured by MTT colorimetric method. 20μl of 5μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-dophenyltetrazolium bromide (MTT) was added to each well. After pre-incubation for 30minutes, the culture medium was replaced with 100μl of isopropanol for the extraction of the dye. The change of the optical density was measured at 570nm by using a 96 well reader.

Result

Table 1. The inhibitory effect on tyrosinase

(%)

No.	Name	1mg/ml	0.1mg/ml
1	Astragali Radix	72.14	24.18
2	Cordyceps sp militaris,	25.11	0.16
3	Cordyceps sp	65.82	-
4	Tribulus terrestris	5.75	-
5	Cuscuta chinensis Lam	6.92	-
6	Bleak beam	19.04	6.46
7	Betula platyphylla var.	-	-
8	Buck wheat	-	-
9	Ephedra sinica Stapf.	0.5mg/ml 81.15	61.25
10	Pinellia ternata Breit.	85.29	9.27
11	Benincasa cerifera Savi.	42.65	6.25
12	Gentiana scabra Bunge	6.27	-
13	Cichorium intybus L.	14.25	12.44
14	Pharbitis nil Choisy	-	-
15	Prunus armeniaca var.	1.8	-
16	Alisma orientalis Juz.	23.08	11.09
17	Sanguisorba officinalis L.	35.09	-
18	Clematis mandshurica Rupr.	17.42	11.76
19	Ginkgo biloba L.	22.28	15.28
20	Kalopanax pictus Nakai	21.76	16.32
21	Ulmus parvifolia Jacq	25.33	-
22	Tricholoma matsutake Sing.	28.57	17.63
23	Dictamnus dasycarpus Turcz.	30.16	14.13

Table 2. The inhibitory effect on elastase

(%)

No.	Name	1mg/ml	0.1mg/ml
1	Astragali Radix	-	-
2	Cordyceps sp militaris,	50.09	3.54
3	Cordyceps sp	-	-
4	Tribulus terrestris	-	-
5	Cuscuta chinensis Lam	-	-
6	Bleak beam	-	-
7	Betula platyphylla var.	52.80	2.71
8	Buck wheat	48.65	5.59
9	Ephedra sinica Stapf.	55.49	-
10	Pinellia ternata Breit.	0.58	0.5
11	Benincasa cerifera Savi.	-	-
12	Gentiana scabra Bunge	0.62	0.51
13	Cichorium intybus L.	5.48	0.71
14	Pharbitis nil Choisy	35.24	10.71
15	Prunus armeniaca var.	18.25	1.09
16	Alisma orientalis Juz.	-	-
17	Sanguisorba officinalis L.	33.44	-
18	Clematis mandshurica Rupr.	-	-
19	Ginkgo biloba L.	39.67	3.12
20	Kalopanax pictus Nakai	-	-
21	Ulmus parvifolia Jacq	11.75	-
22	Tricholoma matsutake Sing.	-	-
23	Dictamnus dasycarpus Turcz.	-	-

Table 3. The scavenging effect on DPPH radical

(%)

No.	Name	0.66 mg/ml	0.066mg/ml
1	Astragali Radix	85.36	20.28
2	Cordyceps sp militaris,	85.38	14.97
3	Cordyceps sp	92.80	19.52
4	Tribulus terrestris	80.16	27.22
5	Cuscuta chinensis Lam	94.77	93.33
6	Bleak beam	87.34	8.55
7	Betula platyphylla var.	90.77	94.46
8	Buck wheat	99.86	98.25
9	Ephedra sinica Stapf.	90.50	76.52
10	Pinellia ternata Breit.	91.94	29.55
11	Benincasa cerifera Savi.	81.89	16.99
12	Gentiana scabra Bunge	71.78	19.86
13	Cichorium intybus L.	88.84	10.89
14	Pharbitis nil Choisy	93.24	79.24
15	Prunus armeniaca var.	49.83	7.46
16	Alisma orientalis Juz.	43.83	11.42
17	Sanguisorba officinalis L.	96.00	92.80
18	Clematis mandshurica Rupr.	88.79	11.52
19	Ginkgo biloba L.	85.35	8.45
20	Kalopanax pictus Nakai	91.27	85.07
21	Ulmus parvifolia Jacq	90.4	90.4
22	Tricholoma matsutake Sing.	90.66	22.15
23	Dictamnus dasycarpus Turcz.	42.42	0.61

Figure 1. The inhibitory effects of other commercially-available compounds on the activity of tyrosinase.

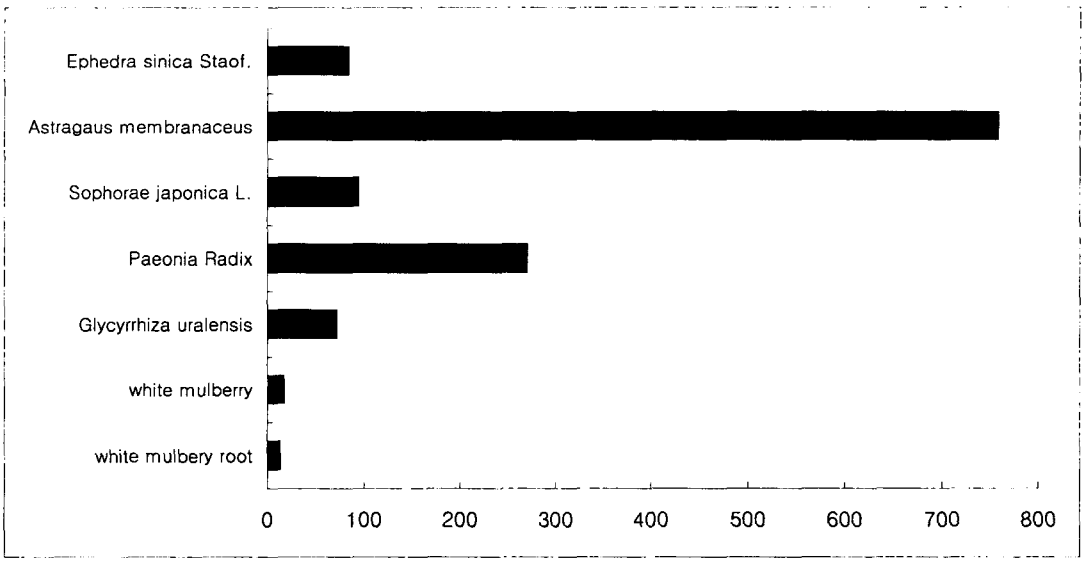


Figure 2. The DPPH radical scavenging effect by other commercially-available compounds

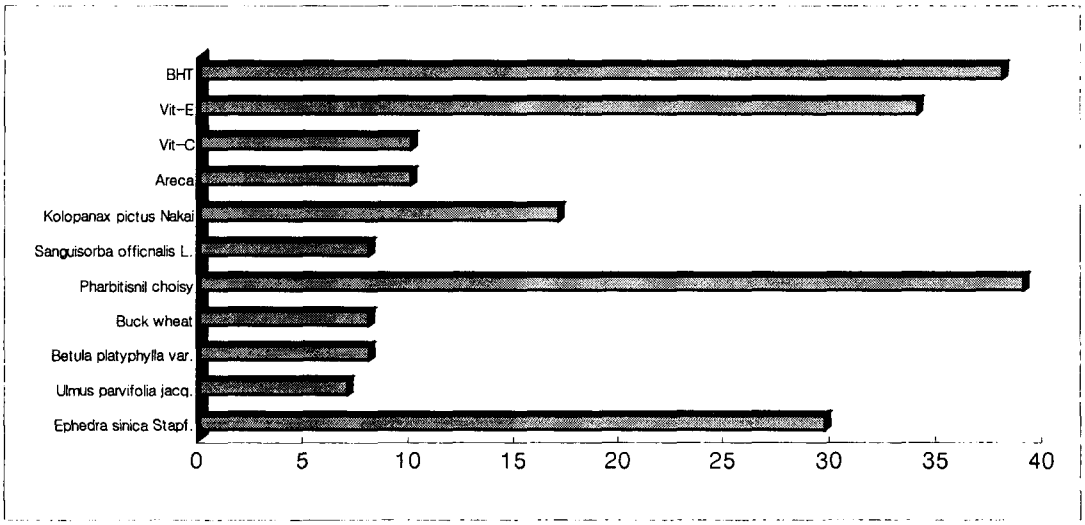
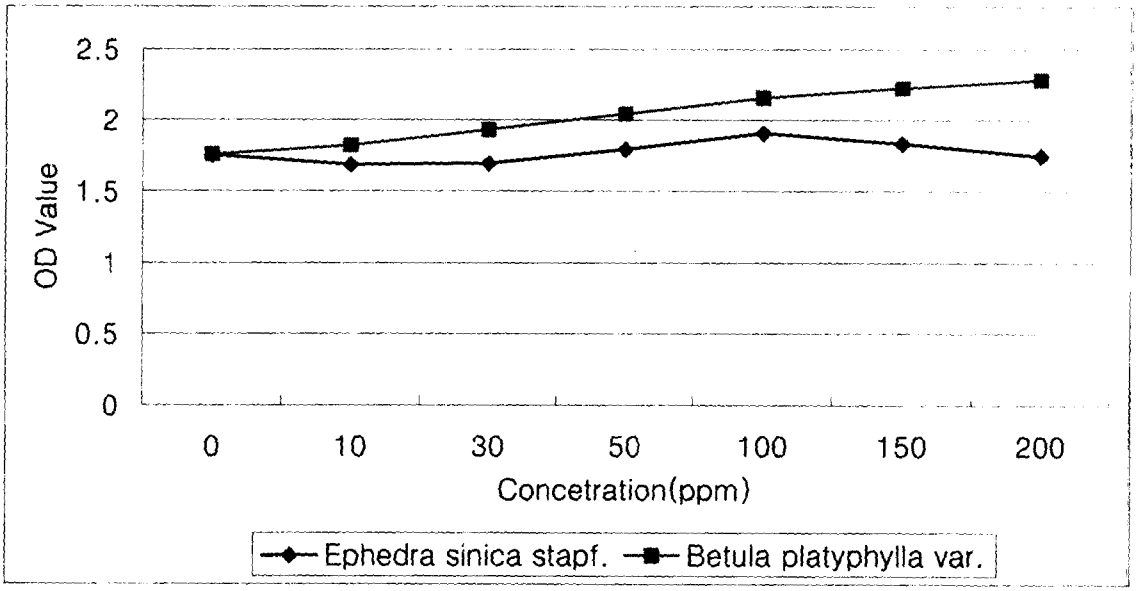


Figure 3. Effect of *Betula platyphylla Var.* and *Ephedra sinsca Stapf* assay with MTT on the cell line, NIH3T3.



Discussion

In this work, an effort to find the natural compounds which have a potential skin-lightening or de-pigmenting, anti-wrinkling and anti-oxidation, with safe and effective ingredients, has been made. Two extracts with positive effects has been found. The extract of *Ephedra sinica stapf* shows an inhibitory effect on tyrosinase (IC_{50} = 83.7μg/ml), on elastase (IC_{50} = 690μg/ml), and also shows free radical scavenging effect (IC_{50} = 29.6μg/ml). The extract of *Betula platyphylla Var.* shows an inhibitory effect on elastase (IC_{50} = 498.1μg/ml), and free radical scavenging effect (IC_{50} = 9μg/ml).

The cytotoxicity of each extract has been examined using the culture of the cell line, NIH3T3. The concentrations of the extracts, applied to the cell cultures, were 10 to 200 μg/ml. No influence has been seen in the application of the extracts.

In the cosmetic, It is thought that the extract of *Ephedra sinica stapf* would be provided a benefit in a skin-lightening and anti-wrinkling agents. Also, The extract of *Betula platyphylla Var.* would be provided a benefit in anti-wrinkling and anti-oxidation agent.

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In vitro Human Skin Model as a Tool for Toxicity and Efficacy Tests of Cosmetics

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Keywords: keratinocyte, fibroblast, toxicity, efficacy, three-dimensional epidermal culture, proliferation, differentiation

Summary

It has been demonstrated in different laboratories that a three-dimensional keratinocyte culture, both morphologically and histologically, resembles human skin *in vivo*. In order to investigate whether the *in vitro* skin models can mimic the functions of skin as well as its morphology and whether they can replace conventional animal models, we set up a range of three-dimensional keratinocyte cultures such as epidermis only, epidermis/dermis and epidermis/dermis/melanocytes. In these models, we were able to control the degree of differentiation and proliferation by differing culture conditions, culture periods and cell densities. It should be noted that using banked keratinocytes at the same passage ascertained the consistent quality of skin models each time. Topical application of cosmetic ingredients on skin models was carried out to determine their toxicity and efficacy through a variety of assays. Cytotoxicity was assessed by both MTT assay and ELISA assay for inflammatory factors. The latter assay is based on the concept that inflammatory factors such as IL-1a and IL-8 are released into the medium from the skin models in responding to foreign materials. Their effects on epidermal proliferation and differentiation were also determined by immunohistochemical staining of the resulting tissue sections for proliferation markers such as Ki-67 and p63 and differentiation markers such as keratin 1/10 and involucrin, respectively. Especially with the skin model containing melanocytes, cosmetic ingredients were tested for their ability to lower the number of melanosome by DOPA or Fontana-Masson staining. From these assays, we were able to select a score of cosmetic ingredients as control materials for further studies. Based on our preliminary data, we would like to report that *in vitro* skin models of three-dimensional keratinocyte culture can functionally and thus physiologically mimic human skin *in vivo*. We are currently in the process of correlating these *in vitro* results with those from animal studies, which will allow us to draw a definite conclusion on a possibility of replacing animal models with *in vitro* skin models.