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Areca catechu L. extract. II. Effects on inflammation and melanogenesis

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Synopsis

Ethanolic extract (CC-516) from *Areca catechu* L. was prepared, and its various biological activities were evaluated. CC-516 showed potent anti-oxidative, free-radical scavenging, and anti-hyaluronidase activity.

The anti-oxidative effect of CC-516 (IC_{50}: 45.4 μg/ml) was lower than that of butylated hydroxytoluene (IC_{50}: 5 μg/ml), but similar to that of tocopherol and higher than that of ascorbic acid. Especially, CC-516 exhibited relatively high free-radical scavenging activity (IC_{50}: 10.2 μg/ml) compared to that of the control. CC-516 effectively inhibited hyaluronidase activity (IC_{50}: 416 μg/ml) and showed inhibition *in vivo* on delayed hypersensitivity as well as croton-oil-induced ear edema in mice when it was topically applied. These results strongly suggest that CC-516 may reduce immunoregulatory/inflammatory skin trouble. Also, from the results, we have elucidated that CC-516 showed anti-allergic and anti-cytotoxicity activity. The whitening effect of CC-516 is shown by the inhibition of mushroom tyrosinase activity with an IC_{50} value of 0.48 mg/ml and of melanin synthesis in B16 melanoma cells. This study indicates that CC-516 is effective on anti-inflammatory activity/anti-melanogenesis, and can be used as a new agent for cosmetics.

INTRODUCTION

Plant sources have been evaluated for developing natural antioxidants and melanogenesis inhibitors that may be involved in anti-aging and skin whitening (1). Many endogeneous plant compounds have been reported to retard the oxidation process in their natural environment (2). Natural antioxidants occur in all higher plants and in all parts of the plant. Typical compounds that possess antioxidative activities include tocopherols, flavonoids, cinnamic acid derivatives, phosphates, and polyfunctional organic acids. Recent studies indicate that the compounds with antioxidative and free-radical scavenging activities can inhibit mutagenesis and carcinogenesis in addition to retardation of aging (1–3). High-molecular-weight hyaluronic acid has an important role in the regulation of scarless repair in fetal wound healing by markedly diminishing the inflammatory response (4). However, degradation products of hyaluronic acid lead to increased inflammation, angiogenesis, fibrosis, and collagen deposition in wound heal-

ing. Recently the harmfulness of ultraviolet (UV) radiation is increasing due to destruction of the ozone layer. Excessive exposure to UV radiation causes postinflammatory pigmentation (4–5). Pigmentary disorders are caused by various factors, including inflammation, the imbalance of hormones, and genetic disorder (6). Melanins play a critical role in the absorption of free radicals and melanogenesis in the skin in a kind of process that produces photoprotective agents against the damaging effect of UV. Many cosmetic and pharmaceutical companies have tried to find an inhibitor of melanogenesis. The regulation of cellular pigmentation can be controlled at many different stages of melanogenesis. Especially, tyrosinase inhibitors and antioxidants can be used for inhibition of cellular pigmentation since the melanin-producing process involves enzymatic and nonenzymatic oxidation reactions. Plant extracts having such biological activities may be a good choice for cosmetic purposes.

We previously screened the inhibitory effects of 150 medicinal plants on elastase activity, and examined their anti-inflammatory effects. The *Areca catechu* methanolic extract showed a high inhibitory effect on elastase and an anti-inflammatory effect, compared to reference compounds (7), and we selected the *Areca catechu* extract as a candidate for new anti-aging and anti-inflammatory agents.

Areca catechu L. is widely cultivated, especially in southern Asia, and its seed is used as a chewing material anthelmintic and also as kompo-traditional medicine. Preparations containing *Areca* are also used as digestive medicines since *Areca* promotes the secretion of saliva (8–9). *Areca catechu* L. contains a number of chemical components, such as alkaloids, tannins, flavonoids, and fatty acids (10,11). To clarity the anti-aging mechanism of *Areca catechu* L. extract against aging, we have studied the anti-inflammatory effect *in vitro* and *in vivo.* Furthermore, the skin-whitening efficacy of the *Areca catechu* extract was also examined by inhibition of mushroom tyrosinase and inhibition of melanogenesis on B-16 melanoma cells. The anti-aging effect of *Areca catechu* L. extract was evaluated by measuring anti-oxidative activity, the free-radical scavenging effect, and inhibition of hyaluronidase *in vitro,* and anti-inflammatory effect/inhibition of delayed hypersensitivity *in vivo.* The safety of CC-516 was evaluated by cytotoxicity on human fibroblasts and skin irritation testing.

MATERIAL AND METHODS

ANTIOXIDATIVE ACTIVITY

A lipid peroxidation system was induced by Fenton's reagent. Each test sample (0.1 ml) and ethyl linoleate (10 µl) were added to an incubation medium (4.89 ml) containing 2% sodium dodecyl sulfate, 1 µM ferrous chloride, and 0.5 µM hydrogen peroxide. The known synthetic antioxidant, butylated hydroxytoluene (BHT) was used as a reference compound. The incubation medium was kept at 55°C for 16 hr. Each reaction mixture (0.2 ml) was transferred into a test tube, followed by addition of 4% BHT (50 µl) to prevent further oxidation. Antioxidative activity of the sample was measured using a thiobarbituric acid (TBA) assay according to the method of Ohkawa *et al.* (12).

FREE-RADICAL SCAVENGING ACTIVITY

Scavenging effect against free-radical generation was measured following the procedure

of Fugita *et al.* (13). The sample solution (2 ml) was added to 2 ml of 60 μM 1.1-diphenyl-2-picryl hydrazine (DPPH) ethanolic solution and kept at room temperature for 30 min. The absorbance was measured at 520 nm.

ANTI-INFLAMMATORY INHIBITION OF HYALURONIDASE

Hyaluronidase activity was determined spectrophotometically by measuring the amount of N-acetylglucosamine formed from sodium hyaluronate (14). Fifty microliters of bovine hyaluronidase (7,900 units/ml) dissolved in 0.1 M acetate buffer (pH 3.5) was mixed with 100 μl of a designated concentration of CC-516 dissolved in 5% DMSO, and then incubated in a water bath at 37°C for 20 min. The control group was treated with 100 μl of 5% DMSO instead of the CC-516. One hundred microliters of 12.5 mM calcium chloride was added to the reaction mixture, and then the mixture was incubated in a water bath at 37°C for 20 min. This Ca^{2+} activated hyaluronidase was treated with 250 μl of sodium hyaluronate (1.2 mg/ml) dissolved in 0.1 M acetate buffer (pH 3.5), and then incubated in a water bath at 37°C for 40 min. One hundred microliters of 0.4 N sodium hydroxide and 100 μl of 0.4 M potassium borate were added to the reaction mixture, and then incubated in a boiling water bath for 3 min. After cooling to room temperature, 3 ml of dimethylaminobenzaldehyde solution (4 g of p-dimethylaminobenzaldehyde dissolved in 350 ml of 100% acetic acid and 50 ml of 10 N hydrochloric acid) was added to the reaction mixture, and then incubated in a water bath at 37°C for 20 min. Optical density at 585 nm of the reaction mixture was measured by using a spectrophotometer. The percentage of inhibition was calculated as:

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

where OD_c is the OD at 585 nm of the control, and OD_s is the OD at 585 nm of the sample.

ANTI-INFLAMMATORY ACTIVITY

For measuring the topical anti-inflammatory activity, the mouse ear edema assay was employed. According to the modified method of Tonnel *et al.* (15), preparations of the CC-516 were topically applied to the right ears of mice (18–22 g) three times at 3-hr intervals. Thirty minutes after the final treatment of the test compounds, 2.5% croton oil or 2% arachidonic acid dissolved in acetone (25 μl/ear) was applied topically to the ears of the mice, and the ear thickness was measured 5 hr after croton oil treatment or 1 hr after arachidonic acid treatment. Percent inhibition of ear edema was calculated by comparison with the control group having the vehicle and anti-inflammatory only. Inhibitory activity against delayed hypersensitivity was measured according to the method of Tarayre *et al.* (16). Briefly, 3% picryl chloride (acetone) was applied to the abdomen of mice (18–22 g). One week later, 3% picryl chloride was applied to the ears of the mice, and ear thickness was measured 24 hr after the picryl chloride solution treatment. Preparations of the test compounds were applied to the ears of the mice daily for 7 days starting from day 0. The differences between the ear thickness of the extract-treated group and the control group treated with picryl chloride and vehicle only were regarded as an indication of inhibitory activity.

INHIBITION OF TYROSINASE

Tyrosinase activity is generally determined by spectrophotometry. The procedure followed that described by Vanny *et al.* (17). The reaction mixture consisted of 0.05 M phosphate buffer (pH 6.8, 2.3 ml), 1.5 mM L-tyrosine solution (0.4 ml), and 2,000 U/ml mushroom tyrosinase (Sigma), in 0.05 M phosphate buffer (pH 6.8, 0.1 ml). A sample solution (0.2 ml) was added to the reaction mixture and incubated at 37°C for 10 min. The optical density at 475 nm was measured by a spectrophotometer (Beckman). The inhibitory activity of the sample was expressed as the concentration of inhibitor (IC_{50}) at which it inhibits 50% of the enzyme activity. The percent inhibition of tyrosinase reaction was calculated as follows:

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

where A is absorbance at 475 nm without a test sample after incubation, and B is absorbance at 475 nm with a test sample after incubation.

INHIBITION OF MELANIN SYNTHESIS IN B-16 MELANOMA CELLS

We examined the inhibition of melanogenesis in B-16 melanoma cells by CC-516 using the modified method of Maeda and Fukuda (18). B-16 melanoma cells (ATCC CRL 6323) were seeded into 60-mm petri dishes at a density of 5×10^5 cells per dish. After the cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l of glucose, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic (Gibco, BRL), the medium was replaced with a fresh medium containing various concentrations of chemicals. Then the cells were cultured for 2 days and the medium was replaced with fresh medium, further incubated for a day. Then cells were harvested with a cell scraper, counted with a haemacytometer, and collected by centrifugation. Melanin was extracted and measured according to the method of Maeda and Fukuda with some modifications (18). Briefly, cell pellets were resuspended in 1 ml of distilled water, frozen at −20°C, and thawed at 37°C. This freezing–thawing process was performed three times. Perchloric acid was added to the cell suspensions at a final concentration of 0.5 N. The tubes were set on ice for 10 min and centrifuged at 15,000 g for 5 min. The pellets were extracted with 0.5 N perchloric acid two times, with cold ethanol/ether (3:1) two times, and with ether. The resulting pellets were dried in air, and 1 ml of 1 N NaOH was added to each tube. The tubes were incubated in a boiling water bath for 10 min to dissolve the pellets. Melanin contents were measured by reading the absorbance at 400 nm, expressed as $A_{400}/10^6$ cells.

CYTOTOXICITY TEST OF CC-516

Human fibroblasts were seeded in a 96-well plate at a density of 10^4 cells/well, supplemented with 0.2 ml of Eagle's minimal essential medium (EMEM) containing 2% FBS, and incubated for 24 hours. After sample addition, the cells were incubated for another 24 hr, and the survival and proliferation of cells were evaluated by MTT assay (19). One-tenth milliliter of MTT solution was added to each well and incubated for 3 hr. After removing the media, 0.5 ml of DMSO was added, and formed formazan was measured by absorbance at 570 nm using an ELISA reader.

HUMAN SKIN IRRITATION TEST

We studied the potential of the CC-516 to irritate human skin in 50 healthy female volunteers using a 48-hr closed patch. No skin irritation occurred after application in 50 volunteers.

RESULTS AND DISCUSSION

We previously screened the inhibitory effect on elastase of methanolic extracts of 150 medicinal plants. The *Areca catechu* extract showed high inhibitory effects comparable to reference compounds (7). For inhibitory effects of several solvent extracts on elastase the ethanolic extract showed the highest effect, and thus CC-516 was used as the sample in this study.

We investigated the effect of CC-516 on the anti-inflammatory and anti-melanogenesis activity by various methods. Table I shows the antioxidative activity of CC-516 and activities of reference compounds such as dl-α-tocopherol, L-ascorbic acid, and BHT. Figure 1 gives a good dose-response relationship between antioxidative activity. BHT appeared to be the most potent inhibitor, with an IC_{50} value of 1.5 μg/ml, while other reference compounds, dl-α-tocopherol and L-ascorbic acid, showed IC_{50} values of 33.6 μg/ml and 219 μg/ml, respectively. The IC_{50} value of CC-516 was 45.4 μg/ml.

Natural anti-oxidants are usually phenolic or polyphenoic compounds, and these compounds include tocopherol, flavonoid, and cinnamic acid derivatives (20). It is known that there are two types of antioxidants (21). The first type of antioxidant inhibits the formation of free radicals that may initiate oxidation. The second type of antioxidant inhibits the free-radical chain propagation reactions. Therefore, some plant extracts may act at the initiation stage of peroxidation, interfering with Fenton's reaction, thus breaking the chain reaction. L-ascorbic acid is one of the most potent scavengers. IC_{50} values of ascorbic acid, dl-α-tocopherol, and BHT were found to be 29.7 μg/ml, 33.5 μg/ml and 37.2 μg/ml, respectively. On the other hand, the IC_{50} value of CC-516 (IC_{50}: 10.2 μg/ml) showed much lower activity than the well-known reference compounds. Free-radical damage to biosystems is one of the major processes that contribute to degenerative diseases like cancer and aging (22). Detailed free-radical mechanisms and their quantitative contributions are still not clear. Despite these uncertainties, it is clear that free-radical scavengers may inhibit endogeneous, metabolically driven, oxidative DNA damage, as well as mutations and aging caused by exogeneous agents (23–25). Most plants have phenolic compounds such as tannins and flavonoids that may contrib-

Table I
Inhibition of Lipid Peroxidation by the CC-516 Measured by Fenton's Reagent Linoleate System and Free-Radical Scavenging Activity of CC-516 in a 1.1-diphenyl-2-picryl hydrazine (DPPH) Free-Radical Generating System

Components	IC_{50} (μg/ml)	IC_{50} (μg/ml)
BHT	1.5	37.2
dl-α-Tocopherol	33.6	33.5
Ascorbic acid	219.0	29.7
CC-516	45.4	10.2

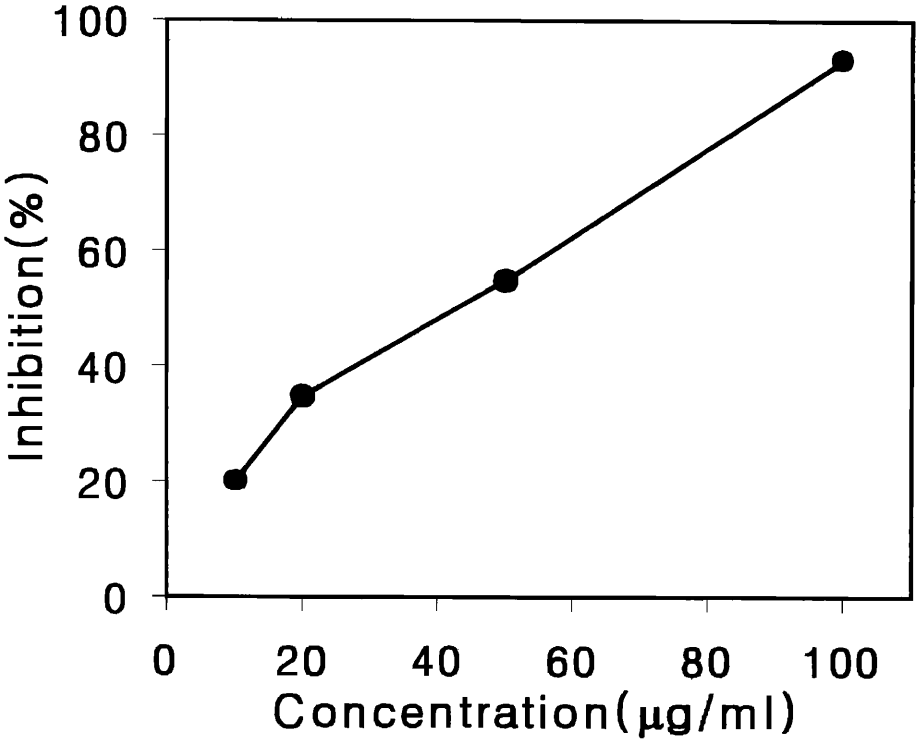

Figure 1. Antioxidative activities of CC-516 in response to CC-516 concentrations.

ute to their antioxidative activities. Masaki (26) reported that *Paeonia suffruticosa* has a strong scavenging activity against DPPH radicals, and he isolated galloyl glucose as an active compound.

In the anti-hyaluronidase activity assay, CC-516 exhibited greater than 65% and 78% of inhibition at concentrations of 0.5 mg/ml and 1 mg/ml, respectively. As shown in Figure 2, CC-516 with 0.1–1 mg/ml as the final concentration exhibited 10% to 78% inhibition. Anti-hyaluronidase activity of *Glycyrrhiza uralensis* as a control exhibited 10–84% of inhibition at a concentration of 0.1–1 mg/ml. IC_{50} values of *Glycyrrhiza uralensis* and CC-516 were found to be 330 ug/ml and 416 μg/ml, respectively. CC-516 contains phenolic compounds such as flavonoids and tannins (27), and thus it could be speculated that the active constituents of CC-516 and *Glycyrrhiza uralensis,* which have inhibitory effects on hyaluronidase activity, are phenolic compounds.

Tables II and III represent the topical anti-inflammatory activity of CC-516. Although the activity was found to be weak compared to the potent activity of the reference compounds, prednisolone and indomethacin, CC-516 showed anti-edematous activity as well as anti-hypersensitivity. The mouse ear edema assay is a frequently used animal model for topical application of the various compounds (28). In croton-oil–induced ear edema, 12-o-tetradecanoylphorbol-13-acetate (TPA) is suggested to produce edema, and leukotriene B_4 is found in ear area. In arachidonic-acid–induced ear edema, eicosanoids such as prostaglandins and leukotrienes are involved in the inflammation. CC-516 did show anti-inflammatory activity against both inflammatory mediators, which sug-

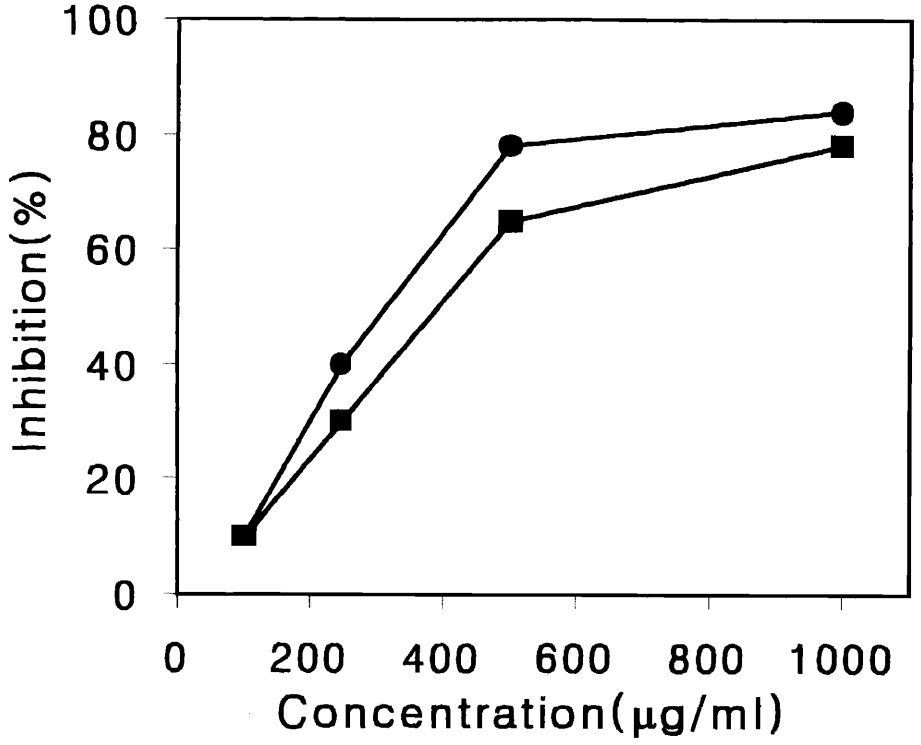

Figure 2. Dose-dependent inhibition of hyaluronidase activities. The effects of CC-516 (■) and *Gylcyrrhiza uralensis* (●) on enzyme activities are indicated as % inhibition compared to the control. Significance of the data is $P > 0.01$. IC_{50} values are 416 μg/ml (■) and 330 μg/ml (●), respectively.

gests that CC-516 might reduce the inflammation in the skin induced by various inducers of inflammation. Because CC-516 also showed inhibitory activity against delayed hypersensitivity, CC-516 may be a useful agent to treat various skin problems.

Previously, several groups, including our groups, demonstrated that flavonoids showed anti-inflammatory activity and anti-allergic activity (29). These results indicate that CC-516 may be, at least in a part, the compound contributing the antioxidative activity *in vitro* and the topical anti-inflammatory activity *in vivo.*

Table II
Mouse Ear Edema Inhibition of CC-516

Groups	Dose (mg/ear)	Thickness increased [mm] (% inhibition)[1]	Thickness increased [mm] (% inhibition)[2]
Control	—	0.25 ± 0.03	0.16 ± 0.03
Prednisolone	0.5	0.16 ± 0.02 (36.0)	NT
Indomethacin	0.5	NT	0.08 ± 0.02 (50.0)
5% CC-516	3 × 50	0.19 ± 0.02 (24.0)	0.14 ± 0.01 (12.5)

[1] Croton-oil–induced ear edema.
[2] Arachidonic-acid–induced ear edema.
NT: not tested.
$P < 0.05$, significantly different from control (n = 5).

Table III
Inhibition of Delayed Hypersensitivity by CC-516

Groups	Dose (mg/ear)	Thickness increased [mm] (% inhibition)
Control	—	0.22 ± 0.04
Prednisolone	7 × 0.1	0.05 ± 0.04 (77.3)
5% CC-516	7 × 50	0.15 ± 0.03 (31.8)

$P < 0.001$, significantly different from control (n = 10).

Table IV
IC_{50} values of Tyrosinase Inhibitors for Mushroom Tyrosinase

Materials	IC_{50} (mg/ml)
Arbutin	6.52
Kojic acid	0.37
Licorice extracts	2.87
CC-516	0.48

Table V
The Effect of CC-516 on Melanin Contents of B-16 Melanoma Cells (n = 3)

CC-516 (mg/ml)	Melanin content (μg/1 × 10^6 cell)	Inhibition of melanogenesis (%)
Control	0.051 ± 0.002	0.00 ± 4.0
0.5	0.026 ± 0.005	49.1 ± 9.8
1.0	0.015 ± 0.003	70.6 ± 6.1
2.0	0.007 ± 0.003	86.3 ± 7.1

Melanogenesis is a series of oxidative polymerization reactions starting from tyrosine and activated by oxidative stress caused by UV. Tyrosinase plays an important role in melanogenesis. Dopaquinone, an intermediate of melanogenesis, is unstable and converted to dopachrome by tyrosinase or autoxidation, and melanin can be formed through the subsequent polymerization reaction. Therefore, inhibition of melanogenesis can be achieved by antioxidation and inhibition of tyrosinase. In fact, kojic acid (29) and arbutin (18), known as tyrosinase inhibitors, have been used in cosmetics for skin whitening.

In this study, the effect of CC-516 on melanogenesis was examined using an *in vitro* enzyme assay and cell culture method. At first, inhibition of tyrosinase-catalyzed dopachrome formation was examined (Table IV). CC-516 (IC_{50}: 0.48 mg/ml) showed stronger inhibition than arbutin (IC_{50}: 6.52 mg/ml) but was weaker than kojic acid (IC_{50}: 0.37 mg/ml). We also examined melanogenesis in B-16 melanoma cells. The results with various concentrations of CC-516 are summarized on Table V. Melanin contents are expressed as $A_{400}/10^6$ cells. CC-516 also showed a strong effect on cultured melanoma cells. The IC_{50} value of CC-516 is about 0.5 mg/ml. From this result, it is deduced that CC-516 may inhibit cellular pigmentation more effectively than arbutin by its antioxidative activity against oxidation of the unstable intermediates of melanin as well as by direct inhibition of tyrosinase. Its inhibitory activity against cellular

pigmentation is thought to be largely due to its antioxidative property that can reduce oxidative stress and inhibit intermediate autoxidation rather than tyrosinase.

The safety of CC-516 was evaluated by cytotoxicity on human fibroblasts and skin irritation testing. In cytotoxicity testing on human fibroblasts, the LD_{50} of CC-516 was about 50 mg/ml, with no change in cell proliferation at a concentration of 50 mg/ml. CC-516 inhibited cellular pigmentation by reducing oxidative stress and inhibiting tyrosinase and autoxidation, and it is expected to have a whitening effect on human skin. Also, CC-516 showed antioxidative activity *in vitro* as well as topical anti-inflammatory activity *in vivo.*

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