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An *in vitro* method for screening skin-whitening products

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Synopsis

Melanoderm (Mat-Tek) is an *in vitro* model of the human epidermis consisting of well-differentiated, cultured human keratinocytes and melanocytes. We utilized this model to evaluate the efficacy, stability, and cytotoxicity of whitening agents. Magnesium ascorbyl phosphate (MAP), kojic acid, and lactic acid in aqueous or anhydrous base were applied to Melanoderm. Following incubation, tyrosinase activity was measured using L-dihydroxyphenylalanine (L-DOPA). Melanocyte staining was observed under the microscope. Melanoderm treated with either MAP, kojic acid, or lactic acid showed 33%, 48%, and 46% reduction, respectively, of tyrosinase activity. Microscopic examination of treated Melanoderm clearly showed the dendritic nature of melanocytes, and normal morphology of keratinocytes and MTT assay suggested that the test materials were not cytotoxic. The kojic acid effect declined with the age of the preparation, and subsequent analysis via high performance liquid chromatography (HPLC) showed kojic acid to be unstable in the aqueous base. Clinical tests using a chromameter to evaluate skin color indicated that kojic acid in an anhydrous base can induce more skin lightening than in the aqueous base. We obtained a good correlation between the Melanoderm, HPLC, and clinical tests. The data show that Melanoderm is a suitable tool for screening whitening agents and developing whitening products. A combination of two *in vitro* tests, such as the Melanoderm and HPLC methods, is useful to evaluate the relative activity, stability, and cytotoxicity of whitening ingredients and products before testing on humans.

INTRODUCTION

Melanin pigments in skin play a key role in determining skin color and are synthesized by large dendritic cells known as melanocytes, which are located at the epidermal–dermal junction (1). Tyrosinase in melanocytes is a key enzyme in the synthesis of melanin pigments (2–4). Melanocytes transfer melanin pigments to neighboring cells such as keratinocytes. Melanin production and transport of melanin is increased by factors such as UV rays, hormones, and chemicals, resulting in darkening of the skin and development of age spots, freckles, melasma, and other disorders of hyperpigmentation (5–8). In Asia, skin-whitening products are very popular and are used to lighten the skin and to treat freckles and skin hyperpigmentation (9–11). The development of successful whitening skin care products depends on the use of effective whitening or depigmenting ingredients that inhibit melanin formation in melanocytes. A number of *in vitro* screen-

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ing methods, such as measuring the inhibition of L-DOPA auto-oxidation (12) and inhibition of tyrosinase and melanin production in melanocytes (13,14) are available; however, these *in vitro* methods are not suitable to measure the activity of whitening ingredients in a finished product. Finished products are cytotoxic to the monolayer cell culture and interfere in L-DOPA auto-oxidation assays. These methods are also limited to compounds that are soluble in water or in culture media. In contrast, a living skin equivalent allows the topical application of a finished product or a water-insoluble compound as it is applied to human skin. Living skin equivalent models are not complete *in vitro* systems, as they lack dermis and other components of human skin such as hair follicles, sebaceous glands, blood vessels, sweat ducts, and sensory nerves. However, these three-dimensional skin models are very useful for testing cosmetics and pharmaceutical products and ingredients before testing on humans. This article reviews a new method to screen tyrosinase-inhibiting agents and subsequently develop whitening products. We utilized a three-dimensional human skin model, Melanoderm, to screen whitening agents before testing the product on humans. Melanoderm is a living skin equivalent *in vitro* model of the human epidermis, consisting of well-differentiated human keratinocytes and melanocytes. The biochemical, histological, and ultrastructural properties of Melanoderm are similar to those of human epidermis. A cross section of Melanoderm shows the presence of stratum corneum, keratinocytes, and dendritic melanocytes localized in the basal cell layer (15). Melanocytes stain positive when exposed to L-dihydroxyphenylalanin (L-DOPA), a precursor of melanin. The relative activity of whitening agents such as kojic and lactic acids and magnesium ascorbyl phosphate (MAP) was measured on Melanoderm. A new method was developed to measure total kojic acid in a finished product using HPLC. Finally, a complete formula was tested on human subjects to determine the correlation with *in vitro* test methods.

MATERIALS AND METHODS

TESTING ON MELANODERM

Melanoderm was purchased from the Mat-Tek Co, Ashland, MA. Kojic acid, lactic acid, and MAP in either an aqueous or anhydrous base or the bases alone were applied to Melanoderm and incubated for two to three days. Tissues were refed with the fresh medium daily. At the end of incubation, the cream was removed. The Melanoderm was first rinsed in phosphate-buffered saline (PBS), treated with 10% buffered formalin for ten minutes at room temperature, and incubated in 0.1% L-DOPA for one hour at room temperature. After one hour Melanoderm was placed in 0.1% fresh L-DOPA and incubated 4 to 16 hours. Melanin from the Melanoderm was extracted and measured at 490 nm using a microplate reader.

HPLC

The kojic acid assay was performed using a Hewlett Packard 1090 model HPLC instrument comprised of an automatic injector, ternary pump, photodiode array detector, and a Chemstation to control the instrument and manage the data. The chromatographic conditions consisted of a C_8, ODS-5, 150 × 4.5 mm column (Metachem #0297) and a

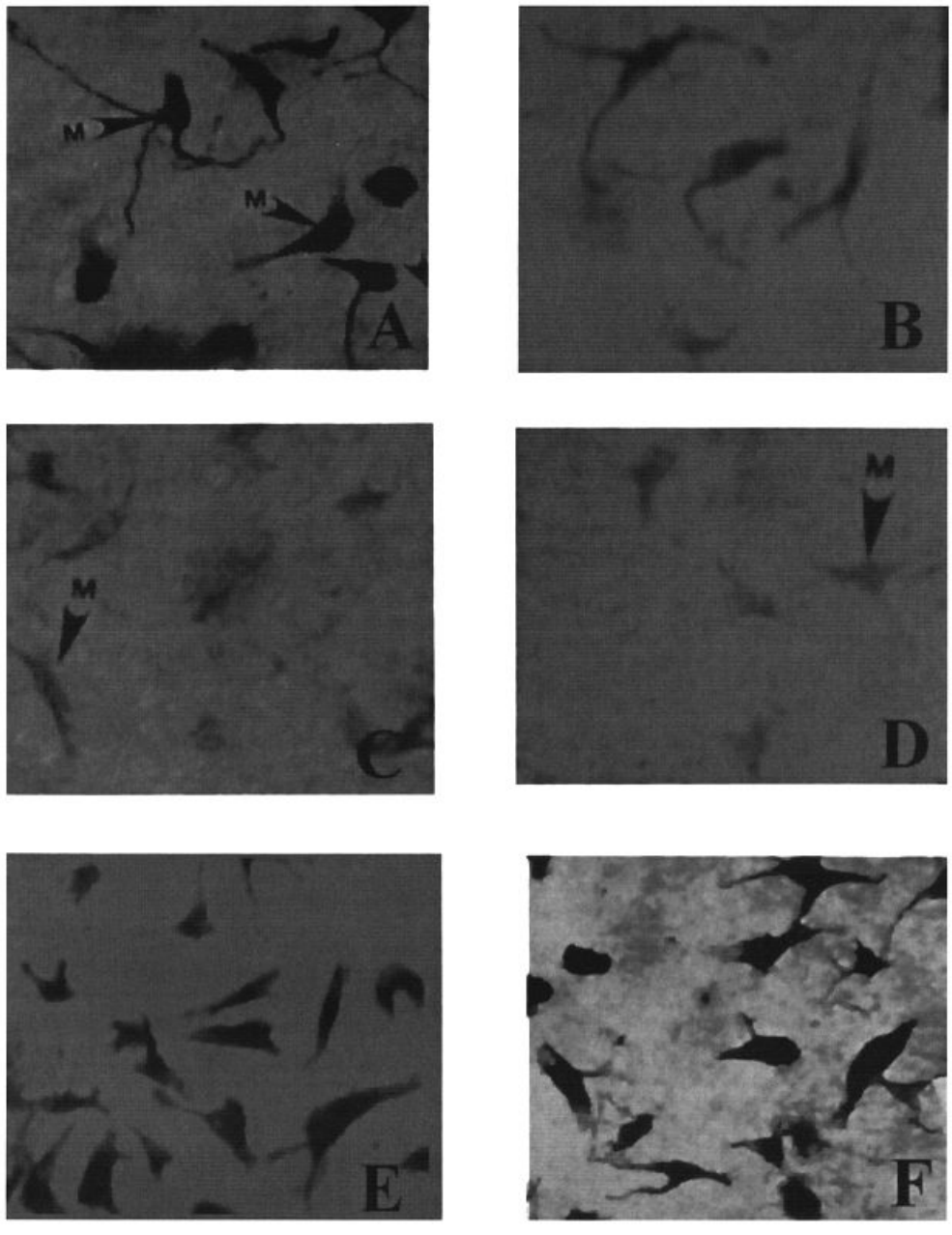

Figure 1. Effect of whitening agents on Melanoderm. Tissues were treated with vehicle control (A), 1% magnesium ascorbyl phosphate (B), 3% lactic acid (C), 1% kojic acid (D), 1% Ascorbic acid (E), and 1% plant extract (F). Tyrosinase activity was detected by L-DOPA as described in Materials and Methods.

mobile phase composed of water:acetonitrile (20:80 v/v). The wavelength of detection was 254 nm. Samples were diluted with water:acetonitrile (50:50 v/v).

CLINICAL TESTING

Ten healthy adult subjects, who gave informed consent, participated in a 12-week study.

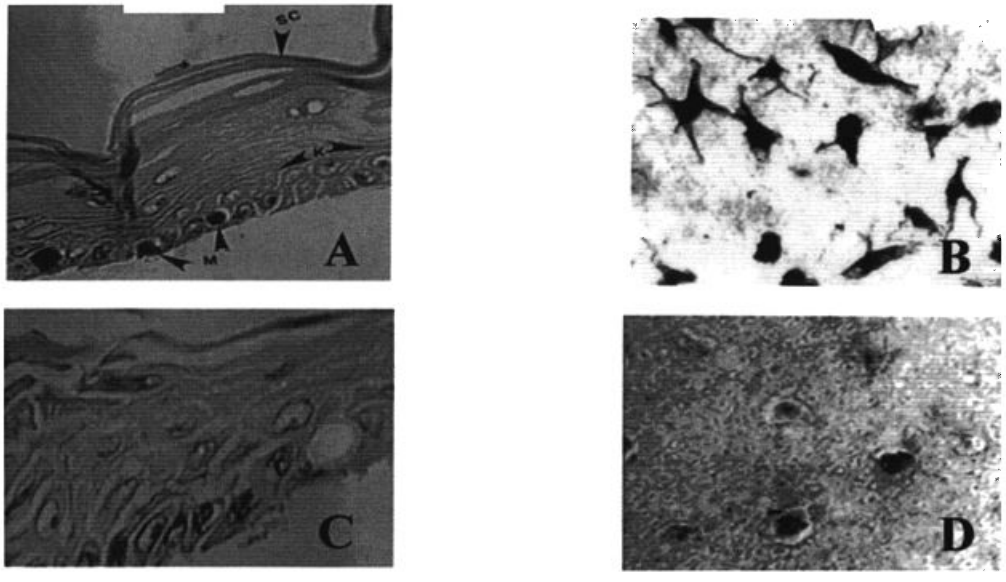

Figure 2. Cytotoxic effect of whitening agent on Melanoderm. A, C: cross sections. B, D: top view. A, B: vehicle control. C, D: whitening agent.

All subjects applied both anhydrous and water-base products containing kojic acid and a retail product containing hydroquinone to their forearms twice daily. Chromameter (Minolta CR200) measurements of skin darkness (L*) were taken at each of the test sites before product application and again 24 hours later.

RESULTS AND DISCUSSION

A variety of whitening agents at different concentrations was tested on melanocytes for an initial screening. After the initial screening, ingredients were selected for further analysis. One percent ascorbic acid, 1% magnesium ascorbyl phosphate, 3% lactic acid, 1% kojic acid, and a plant extract were selected and tested on Melanoderm in a non-ionic aqueous base (Figure 1). After the treatment with whitening agents, Melanoderm was treated with L-DOPA as described in Materials and Methods and examined under the microscope. The intensity of melanocyte staining in Melanoderm depends on the tyrosinase-inhibitory activity of the treated material. Lactic acid (Figure 1C) and kojic acid (Figure 1D) treated samples showed a dramatic decrease in tyrosinase activity as compared to other whitening agents. The MTT assay and dendritic and healthy melanocytes in Melanoderm suggested that these whitening agents were not cytotoxic. However, a whitening agent (Figure 2C,D) showed necrosis of melanocytes and keratinocytes, indicating a cytotoxic effect on Melanoderm. To determine the relative activity of these whitening agents, melanin was extracted from the treated Melanoderm for quantification. A quantitative analysis (Figure 3) showed 48%, 46%, and 33% inhibition by kojic acid, lactic acid, and MAP, respectively. The plant extract did not show any inhibitory activity. Kojic acid was found to be the most effective; however, its activity was lost after a month when it was held at room temperature in the aqueous non-ionic base (Figure 4). To confirm our results and check the stability of kojic acid, we developed a new HPLC

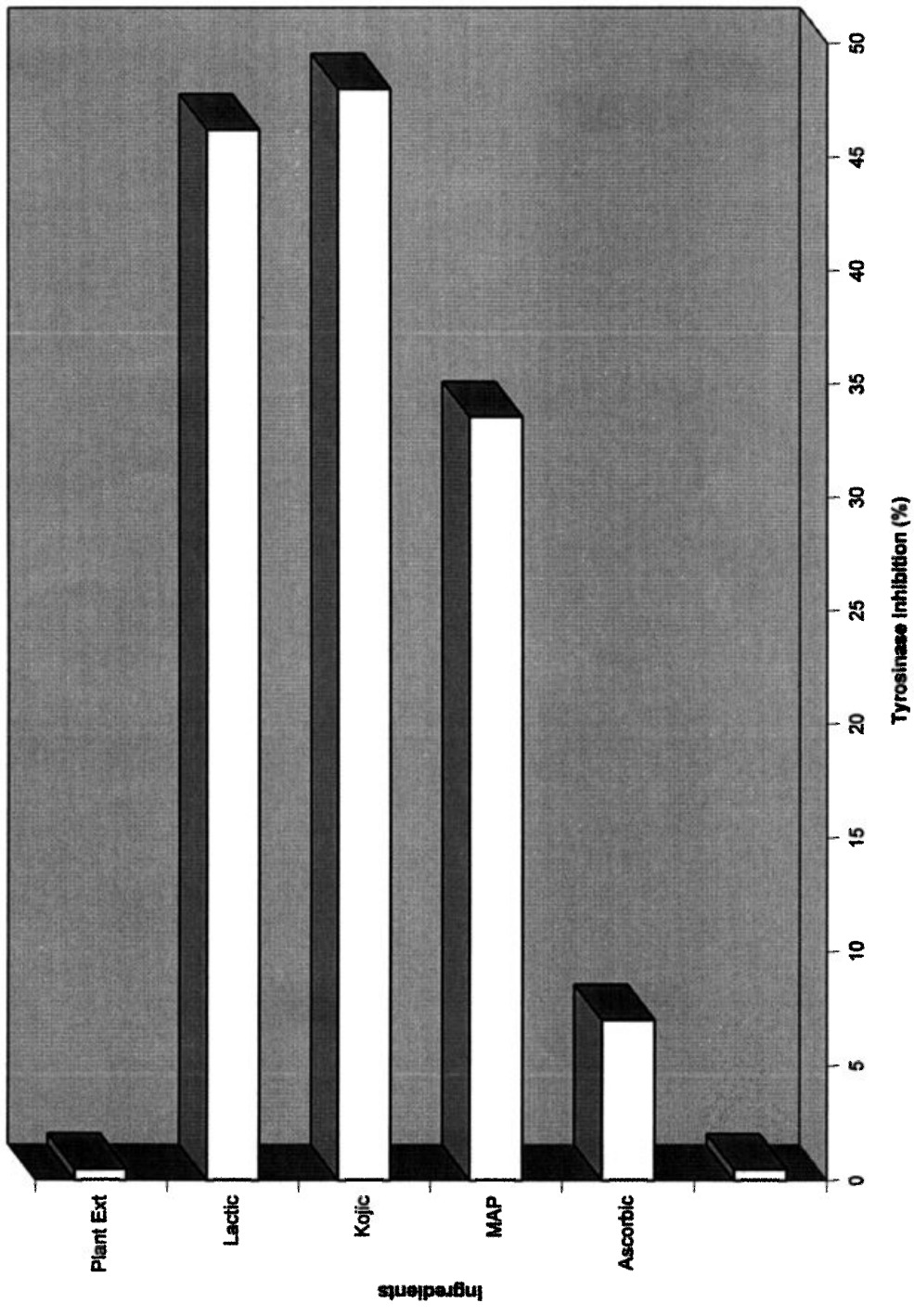

Figure 3. Effect of whitening agents on tyrosinase inhibition in Melanoderm. Tissues were treated with whitening agents. Melanin was extracted from the tissues after L-DOPA treatment.

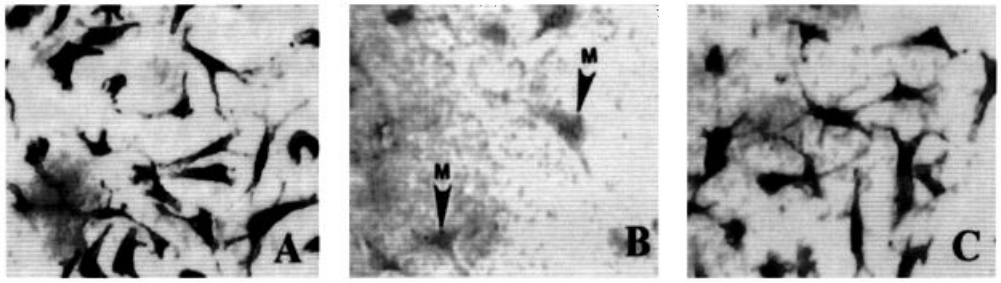

Figure 4. Stability of kojic acid in the aqueous base held at room temperature. Untreated tissues (A) were treated with vehicle control and kojic acid in the aqueous base held at room temperature for one week (B) and one month (C). Tyrosinase activity was detected in melanocytes as described in Materials and Methods.

method to measure kojic acid in different products. Surprisingly, HPLC analysis showed that kojic acid in an aqueous non-ionic base was not stable (Table I). Based on these data, a new anhydrous base was developed and tested on Melanoderm.

Kojic acid in this base showed 41% inhibition in tyrosinase activity (data not shown). Moreover, HPLC data showed that only 2% of kojic acid was lost after 26 weeks in an anhydrous base (Table I). In contrast, 87% of the kojic acid was lost from the aqueous base after five weeks (Table I). To confirm *in vitro* data, clinical testing was conducted with both anhydrous and aqueous bases containing kojic acid. There was a gradual increase in lightening of the skin over a period of three months with the anhydrous base containing kojic acid (Table II). The non-ionic aqueous base containing kojic acid was less effective (Table II), presumably due to loss of kojic acid over a period of three months.

In conclusion, the kojic acid inhibition of tyrosinase in Melanoderm, the kojic acid quantification by HPLC, and the relative clinical efficacy of aqueous and anhydrous bases containing kojic acid showed good agreement. Thus, our present study indicates that Melanoderm can be used as a quick, inexpensive, and reliable *in vitro* model for screening whitening agents and helping to develop whitening products before clinical testing.

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Table I
HPLC Analysis of Kojic Acid

Base	Time (weeks)	Lost (%) 23°C	Lost (%) 37°C
Aqueous	0.00	0.00	—
	2.00	0.00	14.79
	5.00	87.83	86.35
Anhydrous	0.00	0.00	—
	8.00	—	21.22
	12.00	—	19.20
	26.00	2.03	—

Table II
Clinical Study

Type of base with kojic acid	One month	Two months	Three months
Aqueous	0.00	0.90*	0.20
Anhydrous	0.80	1.6*	2.6*
Hydroquinone	1.10*	0.2	2.8*

Caused significant reduction in skin darkness (L*) as compared to baseline value ($P \leq 0.05$)

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