In vitro test method for efficacy evaluation on whitening cosmetics

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Abstract

Various kind of whitening agents have been reported in Korea, but standard efficacy protocols are not established yet. So more economical, reproducible standard efficacy assay for whitening agents are needed. As a dermatology specialist, non radio-labeled intracellular melanin assay may be a good candidate for melanogenesis assay and MTT assay with normal human melanocytes may be a good candidate for cell proliferation assay.

EDUCATION

1974 Hanyang University School of Medicine, Seoul Korea
1988 Ph.D, Hanyang University School of Medicine
1992 Research Fellow in Department of Dermatology, University of Colorado Health and Science Center, Denver. Co, U.S.A

PROFESSIONAL AND RESEARCH EXPERIENCE

1987 Instructor in Department of Dermatology, Soonchunhyang University School of Medicine
1990 Assistant professor in Department of Dermatology, Soonchunhyang University School of Medicine
1995 Associate professor in Department of Dermatology, Soonchunhyang University School of Medicine
2000 Professor and chairman in Department of Dermatology, Soonchunhyang University School of Medicine

PUBLICATIONS

Author or co-author of more than 35 publications in international peer reviewed journals.
Author of Textbook of Dermatology.
Author of more than 75 oral presentations.

국문요약

여러 종류의 미백제들이 한국에서 보고되고 있으나 표준화된 유효성 protocol은 아직 확립되지 않은 실정이다. 그러므로 경제적이고 재현성있는 표준화된 유효성방법이 필요하다. 비방사선표지된 멜라닌 정량법은 멜라닌형성에 대한 정량법으로 좋은 방법이 될 수 있으며 정상적인 사람의 melanocyte를 이용한 MTT assay는 세포의 proliferation을 정량하기 위한 좋은 방법이 될 수 있다.

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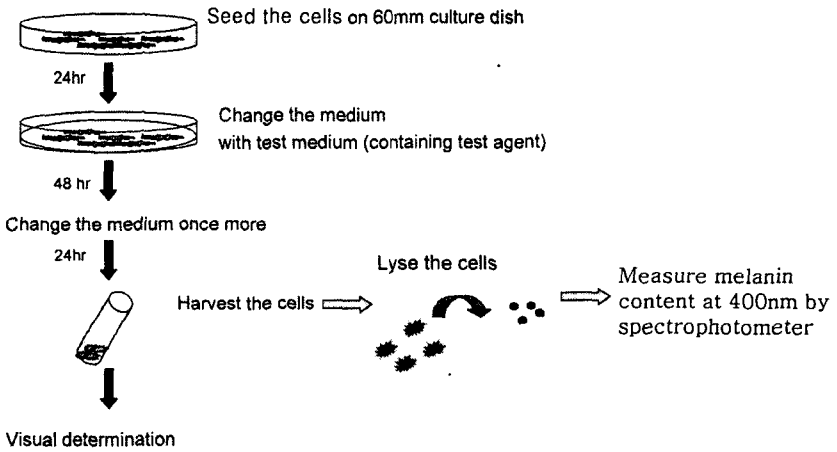
SoonChunHyang University Hospital
Dr. Kyu Wang Whang

1. Assay for melanin(non-radiolabeled cells)

(a) principle of assay

for measurement of intracellular melanin in normal melanocytes or melanoma cell line

(b) method of assay



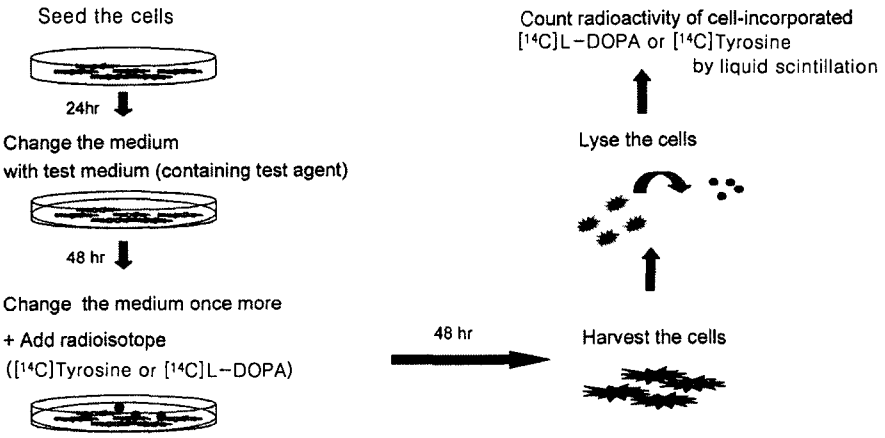
Modified from Siegrist et al. [Analytical Biochemistry(1986)]

2. Assay for melanin(radiolabeled cells)

(a) principle of assay

for measurement of labeled acid-insoluble melanin

(b) method of assay



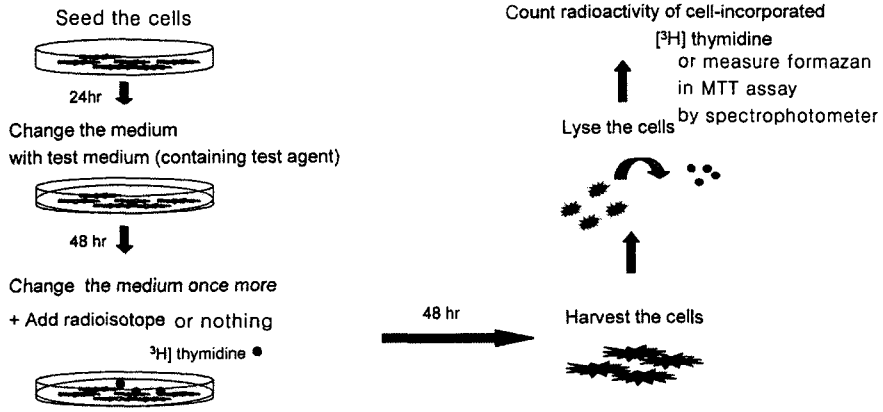
Modified from Hearing. [Methods in enzymology(1987)]

3. Cell proliferation method (Normal Human Melanocyte)

(a) principle of assay

for measurement of melanin index(melanin synthesis/cell proliferation)

(b) method of assay



4. Mushroom tyrosinase assay

$$\text{Tyrosine} \xrightarrow[\text{tyrosinase}]{} \text{L-DOPA} \xrightarrow[\text{tyrosinase}]{} \text{Dopachrome}(A_{475})$$

(a) principle of assay

for measurement of Dopachrome appearance

(b) methods of assay

- tyrosine (enzyme substrate)
- L-DOPA (cofactor)
- 50 mM sodium phosphate buffer
- test agent
- Mushroom tyrosinase (enzyme)

measure at 475nm by spectrophotometer

$$\% \text{ Inhibition} = \frac{\Delta A_{475} \text{ Control} - \Delta A_{475} \text{ Test agent}}{\Delta A_{475} \text{ Control}} \times 100$$

Modified from Vanni et al. [Annali di Chimica(1990)]

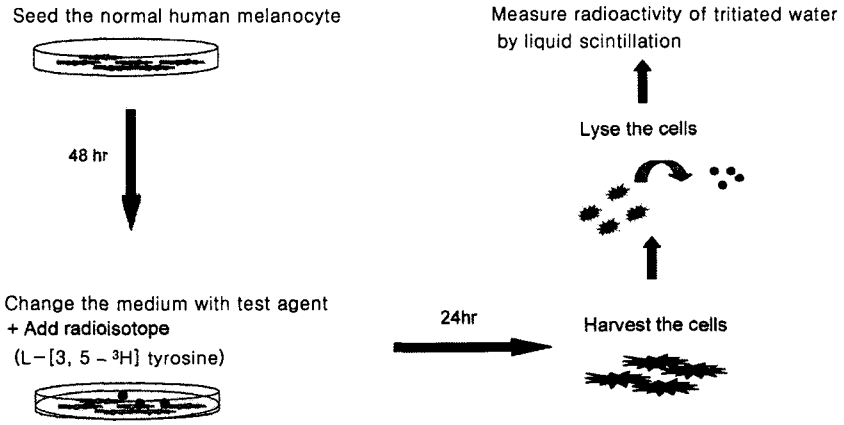
5. In situ Tyrosinase Assay (Tyrosinase hydroxylase Assay)

(a) principles of assay

for measurement of the amount of tyrosinase hydroxylated in living cells

(formation of $[^3H_2]$ from 3-$[^3H]$)

(b) method of assay



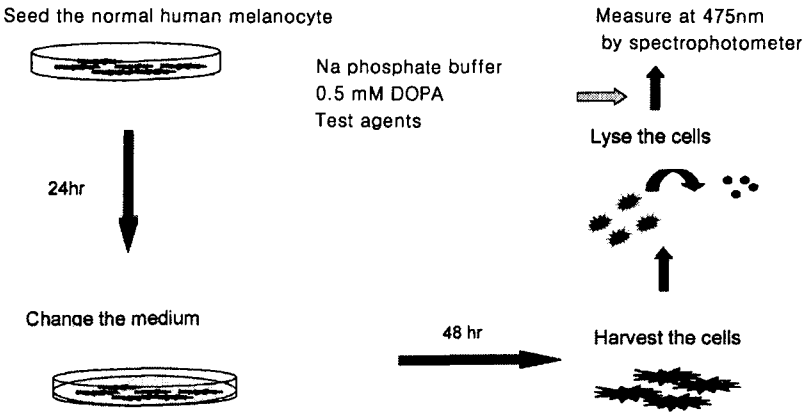
Modified from Pomerantz. [J Biol Chem(1996)]

6. DOPA Oxidase Assay

(a) principles of assay

measurement of DOPA oxidase activity usually carried out by spectrophotometric analysis

(b) method of assay



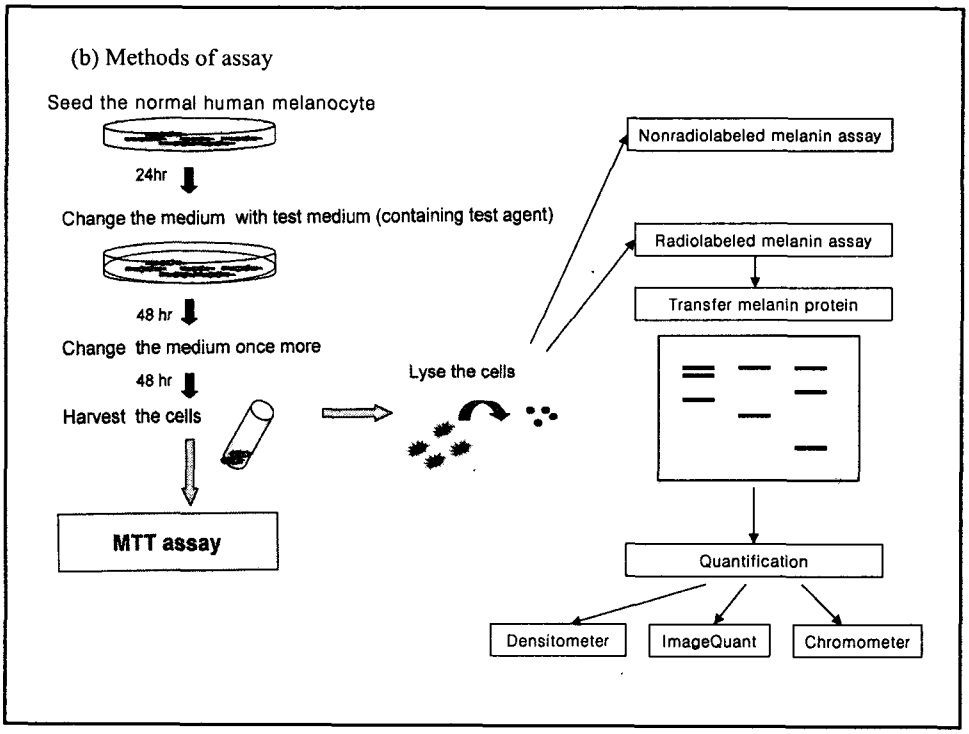
Modified from Hearing. [Methods in enzymology(1987)]

7. STOPR

(Standardized Protocol for Assessing Regulators of Pigmentation)

(a) principles of assay

Reproducible, economical, and reliable series of assays for screening potential melanogenic inhibitors [Virador et al. Analytical chemistry (1999)]



THE EFFECTS OF ENDOTHELIN-1 RECEPTOR ANTAGONISTS (ETRA) ON MELANOGENESIS IN HUMAN MELANOCYTES

INTRODUCTION

Endothelin-1 produced by human keratinocyte acts as a strong mitogen on human melanocyte and has an effects on paracrine linkage between keratinocytes and melanocytes, and plays an important role in UVB induced hyperpigmentation as an intrinsic factor.

In the present study, the role of ET-1 on melanocytes proliferation and melanogenesis was investigated, and the effects of ETRA in cultured melanocyte was also evaluated.

METHODS

1. Cell culture

Normal human keratinocytes and melanocytes obtained from neonatal foreskin were grown in completely MCDB 154 medium (Cascade Biologics, Inc. U.S.A.).

Human melanocytes were seeded at a density of 5 X 10^5 cells/ cm^2, cultured at 37°C under 5% CO_2 atmospherein 6 well plate

2. Addition of ET-1 to medium of melanocytes

ET-1 was added into melanocytes growth media of various concentration (5nM, 10nM, 15nM, and 20nM) respectively. After 3 days cultivation, we washed the cells with phosphate buffer saline(PBS) and collected the cells by trypsinization and centrifugation.

We separated melanin from the pellets of the cells using 5% trichloracetic acid and dissolved the melanin in 1N NaOH solution and determined the melanin contentswith an absorbance at 475nm.

A standard curve for melanin determination was prepared using synthetic melanin(Sigma Chem. Co. Ltd). The cell number was determined with the coulter counter.

3. Inhibition of ET-1 with Endothelin Receptor Antagonists(ETRAs)

We added ET-1 into each well of 6 plate containing melanocyte growth medium in the concentration of 10 nM, which stimulate melanocyte proliferation and melanin synthesis most effectively.

And then five ETRA relatives were added into 10nM of ET-1 contained melanocyte growth media at the concentration of 10nM respectively, the last well contained only ET-1 as a control.

After three days cultivation, melanocyte number was counted and melanin amount was quantified as mentioned above, and the level of cyclic AMP was measured .

4. RT-PCR

(Reverse Transcription-Polymerase Chain Reaction)

We extracted total cellular RNA from ET-1 and ET-1+ ETRAll treated melanocytes.

The PCR cycle was melting for 30 seconds at 94°C , annealing for 30 seconds at 50 °C, extenstion for 50 seconds at 72°C . Total cycle was 33 times.

※ Human Tyrosinase primer
① Forward
5' AGA ATG CTC CTG GCT GTT TTG T 3'
② Reverse
5' GCC ACT GCT CAA AAA TAC TGT C 3'

※ Human β-Actin primer
① Forward
5' CAC CAC ACC TTC TAC AAT GAG C 3'
② Reverse
5' ACT CGT CAT ACT CCT GCT TGC T 3'

MATERIAL

1. ET-1

(human Porcine, Sigma Chemical Co., U.S.A. FW 2491.9)

$NH3^+$-Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-COO^-

(disulfied bonds : between 1 and 15 cystein, between 3 and 11 cysteine)

(---: ET receptor binding site)

2. ET-1 receptor antagonists (ETRA) and fragments

① ETRA I (Sigma Chemical Co., U.S.A)
N-Acetyl-α[10,11-dihydro-5H-dibenzo[a,b]cycloheptadien-5-yl]-D-Gly-Leu-Asp-Ile-Ile-Trp

② ETRA II (Sigma Chemical Co., U.S.A)
N-Acetyl-α[10,11-dihydro-5H-dibenzo [a,b]cycloheptadien-5-yl]-D-Gly-Leu-Asp-Ile-Ile-N-Methyl-Ile-Trp

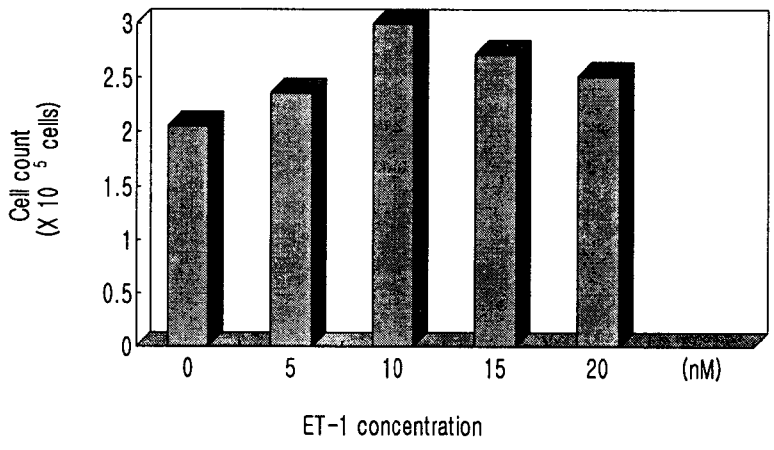
③ ETRA III (Sigma Chemical Co., U.S.A)
N-Acetyl-ß-phenyl-D-Phe-Leu_Asp-Ile-Ile-Trp

④ ETRA IV (Sigma Chemical Co., U.S.A)
N-Acetyl-[D-Trp]-Leu-Asp-Ile-Ile-Trp

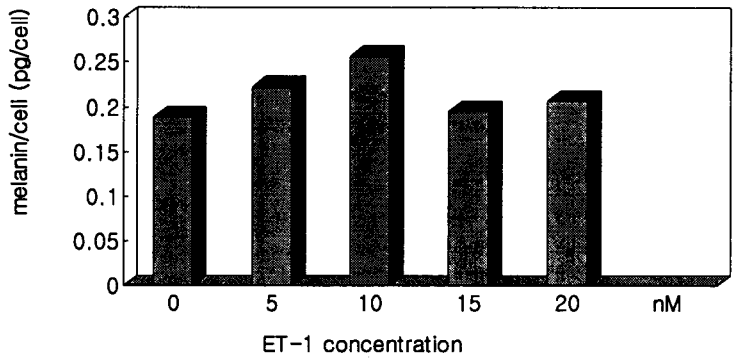
⑤ ETRA V (Sigma Chemical Co., U.S.A)
Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp

RESULTS

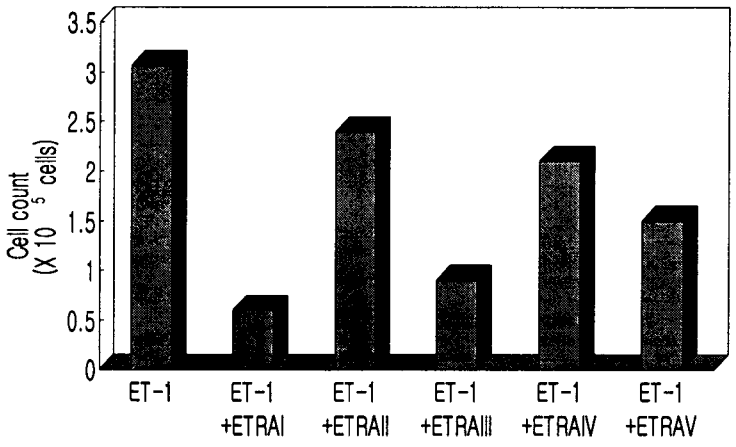
The effect of ET-1 on melanocyte proliferation



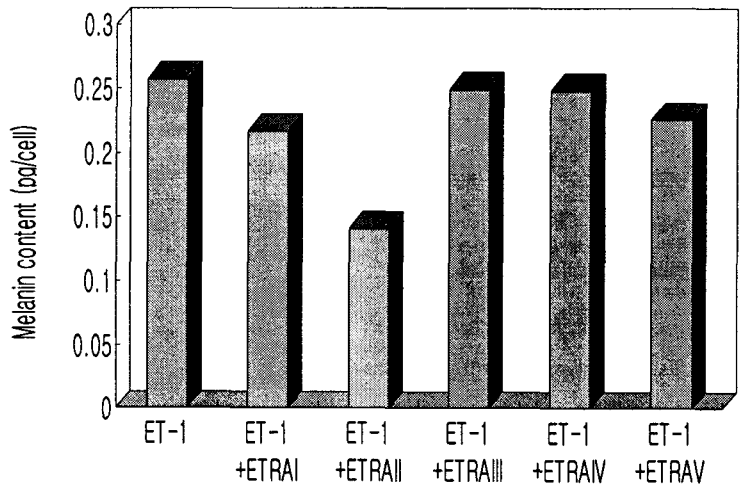
The effect of ET-1 on melanogenesis



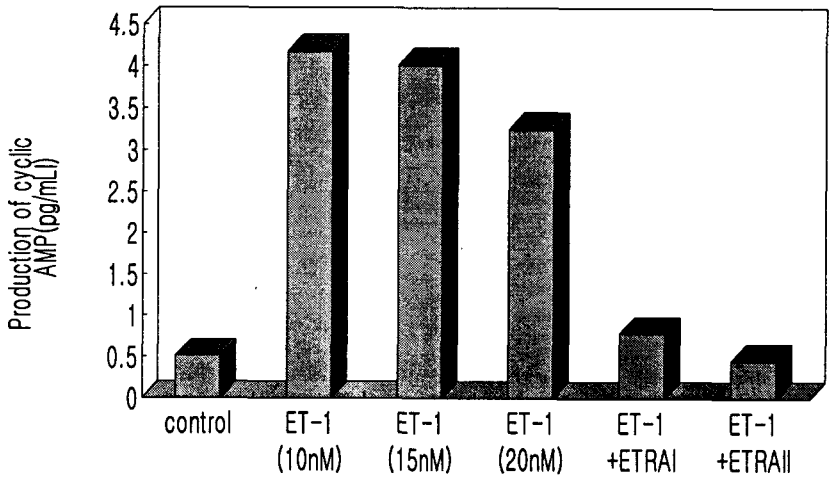
The inhibitory effect of ETRA on melanocyte proliferation

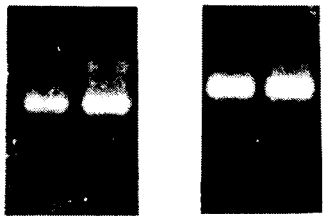


The inhibitory effect of ETRA on melanogenesis



The inhibitory effect of ETRA on melanogenesis





1 2 1 2

Tyrosinase **Human beta-actin**

Fig. 4. The inhibitory effect of tyrosinase synthesis by RT-PCR (1: ET-1 + ETRAII , 2: ET-1)

CONCLUSIONS

We revealed that ET-1 has a strongest stimulatory effect on proliferation and melanogenesis of melanocytes at 10nM.
Also, ETRAs showed inhibitory effects against ET-1.

Among variable relatives of ETRAs, ETRAs which have bulky N-terminal ending have most potent inhibitory effects against ET-1.

We suggest that clinical trial of ETRAs which have bulky N-terminal may be useful in control the pigmentary disorders causing cosmetic problem in Asian.

The Inhibitory Effect of Ramulus ori Extracts on Melanogenesis

Introduction

- The desire to have a more lighter skin in asian women has been tremendous.
- They need very safe whitening products without any irritation. Plants extractable whitening cosmetics meet their needs because they relatively have few side effects.
- Ramus mori(young twigs of Morus alba L) extracts has been known to have whitening effects on korean woman traditionally

Materials

1.Cells

We used cultured normal human melanocytes in MCDB 154 medium.

2.Plants extracts

Ramulus mori extracts powder and its crystallized powder(Mulberrin)

Methods

1.Mushroom tyrosinase assay

Measure tyrosinase activity via dopachrome yield.

2. Melanin assay(non-radiolabeled)

Measure intracellular melanin by spectrophotometer at 400nm

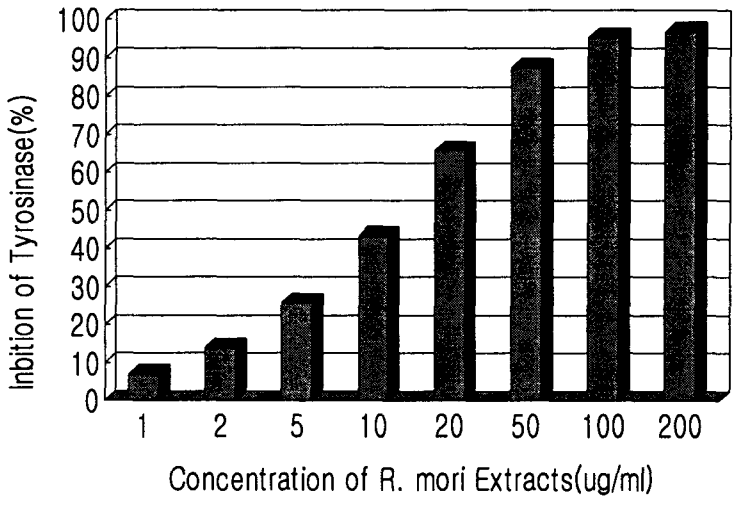
$$\text{Inhibition of melanogenesis \%} = \frac{\Delta A_{400}\,\text{Control} - \Delta A_{400}\,\text{Test agent}}{\Delta A_{400}\,\text{Control}} \times 100$$

3. UV irradiation

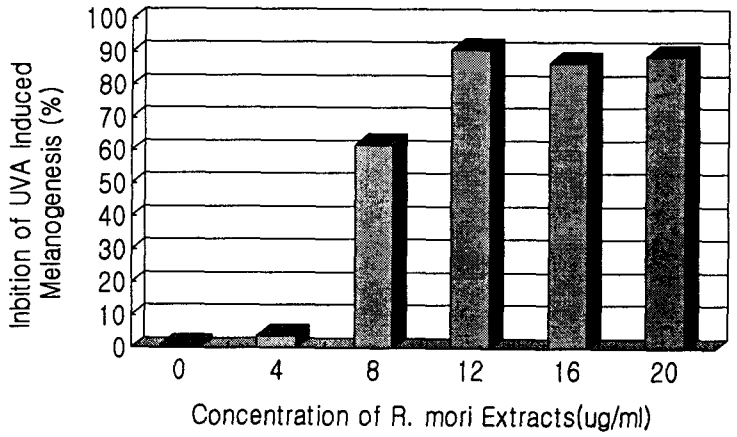
Irradiated UVA on melanocytes in 6 well plates at 10J/cm2.

RESULT

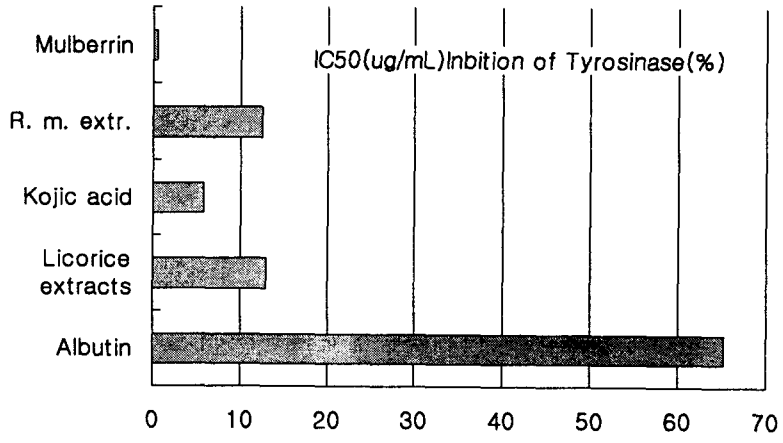
The Inhibitory Effect of Ramulus mori on mushroom tyrosinase



The Inhibitory Effect of Ramulus mori on UVA(10J/cm2) induced melanogenesis



Concentration required for selected tyrosinase inhibitors to reduce mushroom tyrosinase activity 50% (IC_{50})



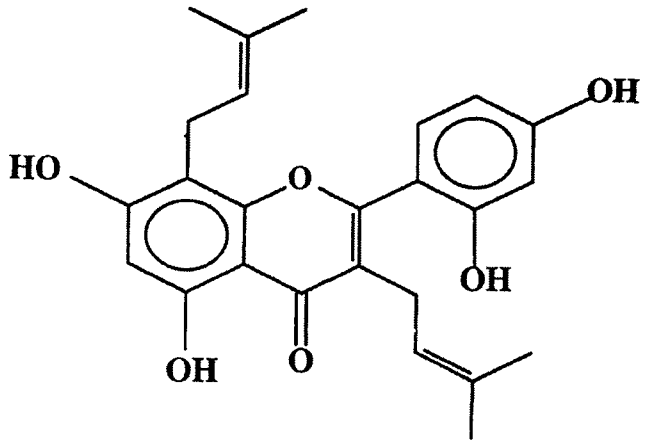


Figure 3.
The structure of 2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3,8-bis (3-methyl-2-butenyl)-4-H-1-bezopyran-4-one isolated from R. mori

Conclusion

1. **Ramulus mori compund(Mulberrin) has strong inhibitory effect on Mushroom tyrosinase at very low concentration.**
2. **Ramulus mori extracts showed potent inhibitory activity against UVA induced melanogenesis.**