Inhibitory Effects of Ramulus Mori Extracts on Melanogenesis

A compound from ramulus mori extracts Inhibits tyrosinase and melanin biosynthesis in melanocytes.

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It has been observed that local increase in melanin synthesis or uneven distribution can cause local hyperpigmentation or spot. Pigmentary disorders are caused by various factors, including inflammation, imbalance of hormones, and genetic disorder[1]. Recently the harmfulness of Ultraviolet(UV) radiation is increasing due to destruction of ozone layer. Excessive exposure to UV radiation causes post-inflammatory pigmentation[2,3]. Most women want to avoid uneven skin pigmentation. To satisfy this desire many cosmetic companies have been developing melanogenesis inhibitors and finding promising active agents for use in cosmetic preparations for skin whitening. In cosmetic preparations, many inhibitors such as kojic acid[4], arbutin[5], ascorbic acid, and licorice extracts6 have been used as whitening purpose. Plant extracts having an inhibitory effect on melanin formation may be a good choice for cosmetic purpose because of their relatively lower side effects. Therefore, we screened 285 plant extracts for their inhibitory activity on tyrosinase[7]. Of the plant extracts, ramulus mori(the young twig of *Morus alba* L.) extracts showed potent tyrosinase inhibition activity. We also identified the active compound in the extract.

Ramulus mori compound

We selected ramulus mori from the results of screening test for tyrosinase

inhibition. Ramulus mori means the young twig of *Morus alba* L. It is commonly found in many parts of the country. We used only the young twig of *Morus alba* L.

Extraction and Isolation : We extracted dried young twig with 70% ethanolic aqueous solution using a vacuum rotary evaporator to concentrate the extract to dryness. To isolate the tyrosinase inhibitor from ethanolic extract, we purified the extract through solvent fractionation, column chromatography, and recrystallization.

The ethanolic extracts were dissolved in water, then shaken with $CHCl_3$, ethyl acetate, and butanol. We purified the ethyl acetate soluble fraction on a silica chromatography and sephadex LH-20 chromatography. We finally isolated the tyrosinase inhibitor by chromatography.

Identification : We crystallized the isolated tyrosinase inhibitor from ether-benzene to yield pale yellowish prisms with a melting point at 148～150℃. The UV λ_{max} of the compound in ethanol were 210nm, 264nm and 315nm. The compound was identified by infra red(IR), mass spectroscopy and nuclear magnetic resonance(NMR) (Table 1-1). We identified the compound as 2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3,8-bis(3-methyl-2-butenyl)-4H-1-benzopyran-4-one, which was found by Nomura et al.[8].

Tyrosinase Inhibition

Tyrosinase activity is generally determined by spectrophotometry. The procedure followed that described by Vanny et al.[9]. For the assay, the test reaction mixture was prepared by adding 0.5㎖ of a solution of the ramulus mori compound, to which 200units of mushroom tyrosinase had been added, to 0.5㎖ of 0.1㎎/㎖ L-tyrosine and 0.5㎖ of 50mM sodium phosphate buffer(pH6.8) of the test mixture. After incubation for 10 minute at 37℃, we measured tyrosinase activity by absorbance at 475nm. We determined the effect of the test compound on tyrosinase inhibition by IC_{50}, the concentration of the compound at which half the original tyrosniase activity is inhibited(Table 1-2).

We calculated the percent inhibition of tyrosinase activity as follows.

$$\% \text{ inhibition} = [(AB)/A] \times 100$$

, where A = absorbance at 475nm without test sample, and B = absorbance at 475nm

with test sample.

Our ramulus mori compound is more potent tyrosinase inhibitior than kojic acid and arbutin. The compound shows a strong inhibitory effect on tyrosinase at very low concentration.

Inhibition of Melanogenesis

We examined the inhibition of melanogenesis in B-16 melanoma cell by the modified method of Oikawa et al.[10]. B-16 melanoma cells(ATCC CRL 6323) were placed in 50mL T-flask at a density of 5.76×106 cells/flask and cultured at 37℃ in Dulbecco's modified eagle's medium(DMEM) containing 4.5g/L of glucose, 10% Fetal Bovine Serum(FBS), and 1% antibiotics. After 48 hours of cultivation, we replaced the medium with new DMEM medium containing ramulus mori compound of various concentration. After 5 days, we washed the cells with phosphate buffer saline(PBS) and collected the cells by trypsinization and centrifugation. We separated melanin from the pellet of the cells using 5% trichloroaceticacid and dissolved the melanin in 1N NaOH solution. We determined the melanin contents with an absorbance at 475nm(Table 1-3). A standard curve for melanin determination was prepared using synthetic melanin(Sigma). The cell number was determined with the coulter counter. Our ramulus mori compound has an inhibitory effect on melanogenesis.

Safety Test

Acute toxicity test : We investigated the potential toxicity of the ramuls mori compound. According to the CTFA Guidelines[11], we assessed acute oral toxicity & acute dermal toxicity of the compound on 60 rats and 24 rabbits respectively. We had examined acute toxicity for 14 days after treatment. No death occurred and abnormality was not detected at clinical findings in rats and rabbits administered orally with the compound. We did not observe changes of body weight in test animals and abnormalities of organ from gross finding of necropsy in test animals(Data not shown).

Skin Irritation test : The primary skin irritation of Ramulus mori compound was investigated to 6 rabbits. We examined skin irritation and clinical signs for 24~72 hours after 7-day treatment using the Draiz's P.I.I.(Primary Irritation Index)[12]. We did not observe side effects, such as erythema, eschar and edema.

Eye irritation test : The potential toxicity of the compound was determined according to AFNOR(Association Francaise de Normalization) guidelines in an eye irritation test on 9 rabbits. We examined both eyes at 1hours, Days 1, 2, 3, 4 and 7 after treatment. We did not see any clinical signs.

Skin Sensitization test : The sensitization potential of the compound was assessed in 33 guinea pigs by the methods of Magmusson & Kligman Maximization Test[13]. We had no observed skin responses in test animals.

Human skin irritation test : We studied the potential of the compound to irritate human skin in 50 healthy female volunteers using 24 hours closed patch. No skin irritation occurred after application in 50 volunteers.

Conclusions

We found that ramulus mori extracts had a strong inhibitory activity against melnogenesis and the active compound was 2-(2,4-Dihydroxyphenyl)-5,7-dihydroxy-3,8-bis(3-methyl-2-butenyl)-4H-1-benzopyran-4-one. The results of safety tests showed the compound to have no irritation and sensitization potential. Conclusively, ramulus mori compound is strongly expected to be an effective ingredient for the improvement of freckles and melasma.

References

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Table 1-1. Data from spectroscopic studies

IR λ max cm^{-1} (KBr) I	3370, 1660, 1630, 1610,1560
Mass m/z II	422, 407, 379, 367, 323

1H NMR(CD_3COCD_3) δ, ppm

1.43(3H, s, C11-CH_3), 1.57(9H, s, C11-CH_3 and C14-$CH_3 \times 2$),
3.12(2H, brd, J = 8Hz, C9H$\times$2), 3.35(2H, brd, J = 8Hz, C12-H $\times$2),
5.20(2H, m, C10 and C13 - H), 6.31(1H, s, C6-H),
6.43(1H, dd, J=2 and 8Hz, C5'-H), 1.52(1H, d, J = 2Hz, C3'-H),
7.20(1H, d, J = 8Hz, C6'-H), 13.05(1H, s, OH)

13C NMR(DMSO-d_6) δ, ppm

C-2(159.1), C-3(119.6), C-4(182.1), C-4a(103.4)
C-5(155.1), C-6(98.2), C-7(161.9), C-8(105.6)
C-8a(160.2), C-9(23.4), C-10(121.9), C-11(131.6)
C-12(25.6), C-13(17.4), C-14(21.2), C-15(122.3)
C-16(130.9), C-17(25.6), C-18(17.4), C-1'(111.6)
C-2'(156.8), C-3'(102.8), C-4'(161.6), C-5'(106.8), C-6'(131.6)

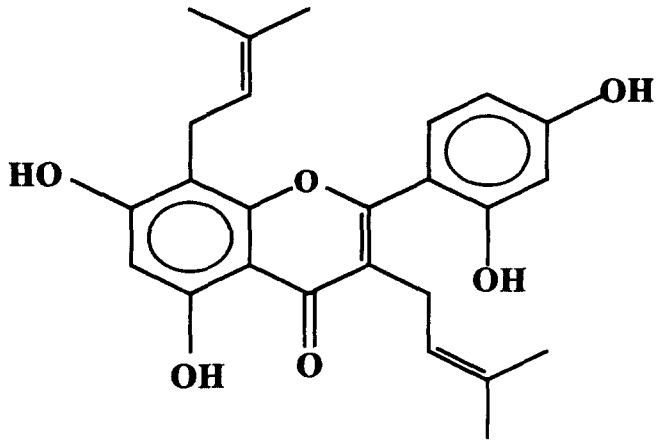

Figure 1-1. The Structure of 2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3,8-bis(3-methyl-2-butenyl)-4H-1-benzopyran-4-one isolated from ramulus mori

Table 1-2. Comparison of tyrosinase inhibitors for mushroom tyrosinase : IC_{50}

Materials	*IC_{50}(μg/mℓ)*
Arbutin	65.2
Licorice extracts	12.88
Kojic acid	5.82
Ramulus mori extracts	12.48
Ramulus mori compound	0.507

Table 1-3. The effect of ramulus mori compound on melanogenesis in B-16 melanoma cells

Ramulus mori compound (μg/mℓ)	*Melanin content (pg/cell)*	*% Inhibition of Melanogenesis*
Control	4.281 ± 0.172	0.00 ± 4.02
10	1.953 ± 0.493	54.38 ± 11.52
20	1.426 ± 0.398	66.69 ± 9.30
50	1.072 ± 0.281	74.96 ± 6.56
100	0.465 ± 0.305	89.13 ± 7.12