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DEVELOPMENT OF NEW WHITENING AGENT. THE INHIBITORY EFFECTS OF LAGENARIA LEUCANTHA ON MELANOGENESIS AND DEPIGMENTATION EFFECT OF GOLD FISH

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ABSTRACT

In this study, we demonstrated the whitening effect of *Lagenaria leucantha* through the melanin biosynthesis of *S bikiniensis* and inhibition of melanogenesis in cultured B16 melanocytes. And we confirmed the whitening effect of *Lagenaria leucantha* through the depigmentation of gold fish in vivo. The melanogenesis of B_{16} melanocytes was founded to be activated dose and time dependently by the treatment of α- MSH. When the B_{16} melanocytes was treated with 200nM of α - MSH, the morphology of melanocytes was remarkably changed. The melanin content and the synthesis of tyrosinase were strikingly increased. *Lagenaria leucantha* inhibited the melanin formation stimulated by α- MSH without affection of cell viability. However, *Lagenaria leucantha* didn't inhibit tyrosinase activity and showed weak suppression on the synthesis of tyrosinase. These results suggest *Lagenaria leucantha* might inhibit melanin formation with tyrosinase independent manner. *Lagenaria leucantha* also inhibited melanin biosynthesis with 18mm inhibition zone in *S.bikiniensis*. To evaluate the inhibitory activity of melanogenesis of *Lagenaria leucantha* in vivo, we examined its effect on depigmentation of gold fish. *Lagenaria leucantha* remarkably reduced the size and density of melanophores in gold fish. These results suggest that *Lagenaria leucantha* can be used as a whitening agent in cosmetics.

INTRODUCTION

Melanins are the pigments that impart color to our skin, hair and eyes. Melanins also absorb the harmful ultraviolet(UV) spectra and protect us from UV-induced skin cancers and skin photoaging. However, hyperpigmentation such as freckles and brown spots can be a serious aesthetic problem in Asia. Because many Asian women want to have lighter skin. To develop whitening agents, we have been studying many kinds of plant extract. In this study, we examined the whitening effect of the *Lagenaria leucantha* extract. Melanocyte-stimulating hormones(MSH) play a key role in the regulation of pigmentation throughout the vertebrates. It has been reported that human being are injected with MSH, or peptides closely resembling MSH, melanin production in their skin is accelerated to a rate similar to that seen following exposure to sunlight(I). On the basis of this report, we stimulated B_{16} melanocytes with synthetic α - MSH. And we examined the suppression of *Lagenaria leucantha* on the melanogenesis induced by α - MSH. We also examined the inhibitory effect against melanin biosynthesis of *S.bikiniensis*. The gold fish is sensitive to the inhibition of the melanogenesis. We

could observe not only the change of the appearance but also the melaninphores on the scale from the gold fish.

MATERIALS AND METHODS

Preparation of *Lagenaria leucantha* extract

Lagenaria leucantha was cultivated in chungbuk province in Korea. *Lagenaria leucantha* was immersed in ethanol for 10 days. After removing the *Lagenaria leucantha*, the filtrate was concentrated under reduced pressure. Then, *Lagenaria leucantha* extract was partially purified using centriprep (Amicon,Inc.,Beverly, USA).

Effects of α - MSH on melanogenesis in B_{16} melanocytes

B_{16} melanocytes were placed in a 25 cm^2 culture flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum(FBS) and 200nM of synthetic α - MSH(Sigma Chemical Co., St Louis, Mo, USA) at a density of 3 x 10^5 cells/tlask and cultured at 37 C in a full humidified atmosphere composed of 5% CO_2, for 48 hr. The cells were harvested with trypsinization and pelleted by centrifugation at 1,000rpm for 10 min. The color of the cell pellet was assesed visually. The cell number was determined by MTT assay, as described by Mosman(2). It has been reported that tyrosinase is synthesized in B_{16} melanocytes within 24hr(3,4). On the basis of these reports, we evaluated the effect of α–MSH on tyrosinase synthesis in B_{16} melanocytes. As an indication of tyrosinase synthesis, we used the tyrosinase activity. Tyrosinase activity was measured by the method of Maeda *et al*(5), which we modified(6). After washing with PBS, the cells were lysed with 1 ml of sodium phosphate buffer(0.1M, pH6.8) containing 1% Triton X-100 at room temperature. Then, the cell lysates were centrifuged at 2,000rpm for 20 min. We got the supernatant as enzyme source. 100 μl of sodium phosphate buffer containing 0.1% L-Dopa was added to 50 μl of the enzyme fraction and incubated at 37 C for Ihr. We measured enzyme activity with absorbance at 490nm. Melanin content was determined according to the method of Oikawa et al(6). The cells were lysed with the method described in the previous. The cell lysates were centrifuged at 3,000rpm for 20min. The pellet was dissolved with 1N NaOH. The melanin content was quantified with an absorbance at 490nm. A standard curve for melanin determination was prepared using synthetic melanin(Sigma Chemical Co., St Louis, Mo, USA).

Evaluation of *Lagenaria leucantha* on melanogenesis in B_{16} melanocytes

2 x 10^6 cells were placed in 75 cm^2 culture flask in DMEM containing 10% FBS, 200nM of α -MSH and *Lagenaria leucantha* and cultured at 37 C in a full humidified atmosphere composed of 5% CO_2 for 3days. The intracellular melanin content and cell number were determined with the method described in the previous.

Evaluation of *Lagenaria leucantha* on synthesis of tyrosinase in B_{16} melanocytes

2 x 10^6cells were placed in 75 cm^2 culture flask with DMEM containing 10% FBS, 200nM of α -MSH , sample and incubated at 37 C in a full humidified atmosphere composed of 5% CO_2 for 3 days. The tyrosinase activity was measured with the method described in the previous.

Evaluation of *Lagenaria leucantha* Inhibition of melanin production of *S.bikiniensis*

A preserved culture of *S.bikiniensis* NRRL B- 1049 (KCTC-9172) was inoculated on a papavizas' VDY A agar slant which contained V -8 juice (Campbell Soup Co.) 200 ml, glucose 2g, yeast extract (Difco Co.) 2g, CaCO3 1g, agar (Difco Co.) 20g, and distilled water 800 ml, the pH being adjusted to 7.2 before autoclaving. After incubating at 28 C for 2 weeks, 2 ml of sterile water was added onto the slant culture and spore mass formed on the aerial mycellium was scraped with an inoculating loop. The spore suspension thus obtained was transferred to a sterile test tube. Agar medium ISP No.7 supplemented with 0.2% Bacto-yeast extract (Difco Co) was poured into petri dishes (90MM-diameter). After solidification, 0.4ml ofthe spore suspension of *S.bikiniensis* was added to the agar plate and spread over the agar surface uniformly with glass hockey bar. After drying of the agar surface, a paper disc (8mm diameter) soaked with sample solution was placed on the agar plate. The plate was incubated 28 C for 48hr, the resulting zone of inhibition of melanin formation was measured from the reverse side ofthe plate. 4-hydroxyanisole was used as a reference standard.

Evaluation of *Lagenaria leucantha* on depigmentation of black gold fish

Black gold fish were raised in water containing 1mg/ml of *Lagenaria leucantha*. After 10 days, 20 days and 30 days, the scale from the gold fish was observed using image analyzer.

RESULTS

Effects of α -MSH on melanogenesis in B_{16} melanocytes

The treatment of α -MSH results in remarkable change of morphology in B_{16} melanocytes. (Fig.1) α -MSH also increased intracellular melanin content and the synthesis oftyrosinase. At a concentration of 200nM, the α -MSH treated cells have intracellular melanin 5 times as much as control. The α -MSH treated cells also were increased the synthesis of tyrosinase to 98 10% compared to control cells (Fig.2).

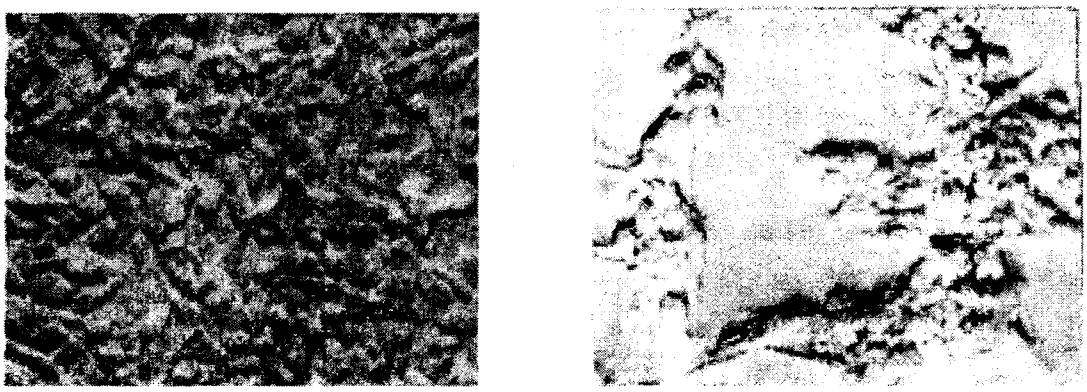


Fig. 1 Effect of α -MSH on melanogenesis in B_{16} melanocytes
left: control *right: 200nM of α -MSH*

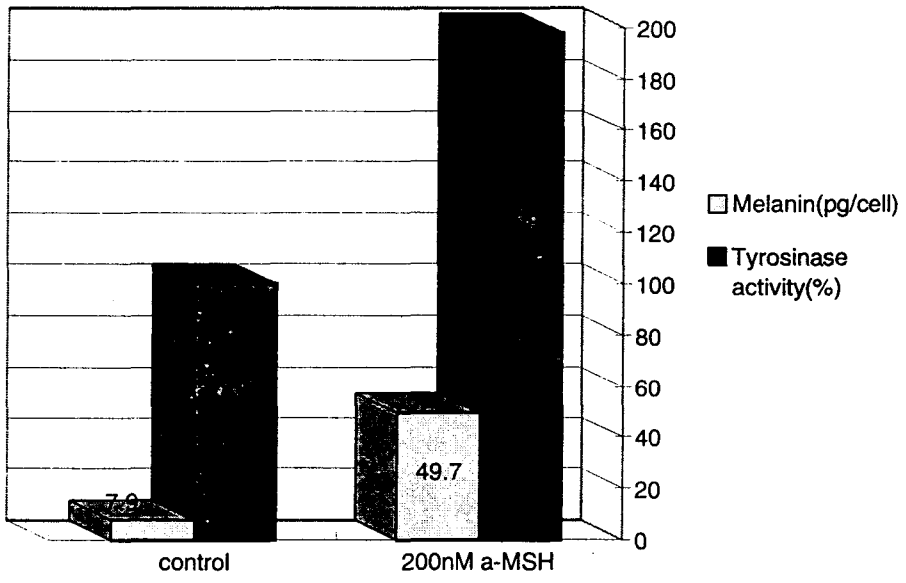
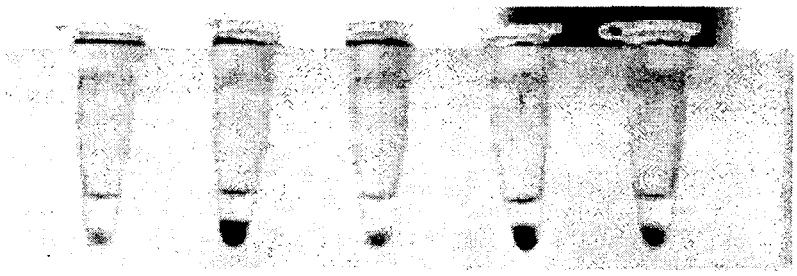


Fig. 2 Activation of melanin formation by the treatment of α–MSH
B_{16} melanocytes were cultured with α–MSH for 2 days.

Effect of *Lagenaria leucantha* on melanogenesis in B_{16} melanocytes

We quantitatively examined the effect of *Lagenaria leucantha* on melanogenesis. *Lagenaria leucantha* decreased the intracellular melanin content (Fig.3). 1.2mg/ml of *Lagenaria leucantha* decreased the melanin content to 80% 10% compared to that of control cells (Fig.4). In these experimental conditions, *Lagenaria leucantha* did not have any cytotoxic effects.



1 2 3 4 5

Fig. 3 Effect of Lagenaria leucantha on melanogenesis in B_{16} melanocytes
B_{16} melenocytes were cultured with α-MSH and sample for 3 days.
1: Arbutin (0.4 mg/ml) 2: Control (PBS) 3: Kojic acid (0.4 mg/ml)
3: Lagenaria leucantha (1.2 mg/ml, M.W.>3,000)
4: Lagenaria leucantha (1.2 mg/ml)

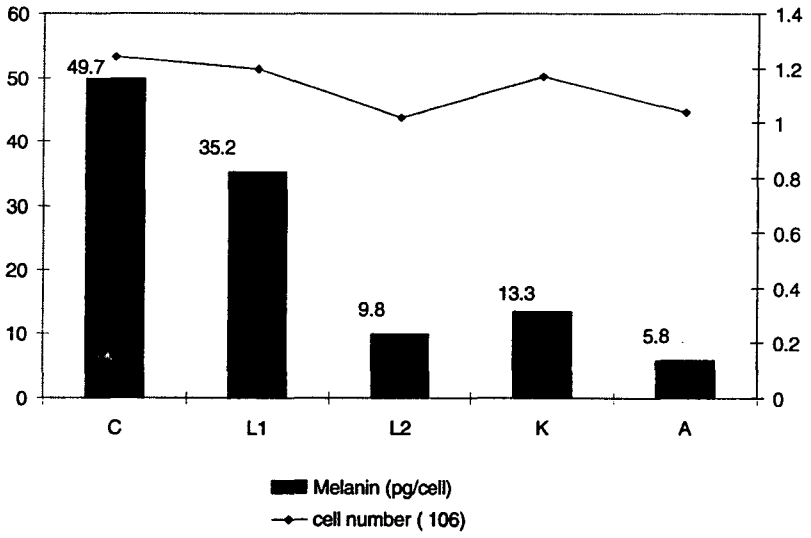


Fig. 4 Effect of Lagenaria leucantha on melanin formation
B_{16} melanocytes were cultured with α-MSH and sample for 3 days
C: Control L1: Lagenaria leucantha (1.2 mg/ml)
L2: Lagenaria leucantha (1.2 mg/ml, M.W. >3,000)
K: Kojic acid A: Arbutin

Effect of *Lagenaria leucantha* on the synthesis of tyrosinase in B_{16} melanocytes

As an indication of tyrosinase syhthesis. We examined tyrosinase activity in B_{16} melanocytes cultured in the presence of sample. *Lagenaria leucantha* decreased the synthesis of tyroinase only 18% compared to that of control cells (Fig.5)

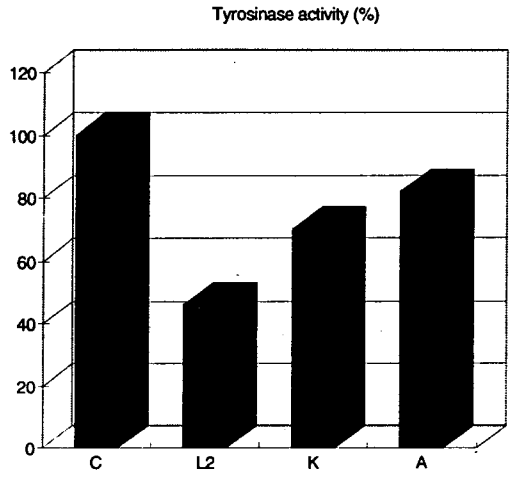


Fig. 5 Evaluation of Lagenaria leucantha on the synthesis of tyrosinase
B_{16} melanocytes were cultured with α-MSH and sample.
C: Control K: Kojic acid A: Arbutin
L2: Lagenaria leucantha (1.2 mg/ml, M.W. >3,000)

Effect of *Lagenaria leucantha* on melanin biosyhnthesis of *S.bikiniensis*

Lagenaria leucantha inhibited melanin formation of *S.bikiniensis* with 18mm inhibition zone (fig 6).

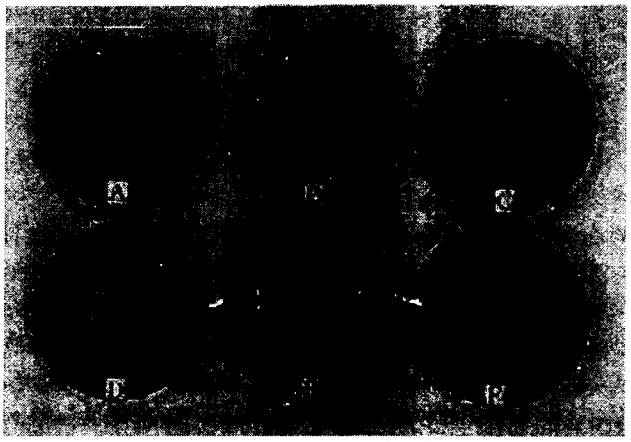
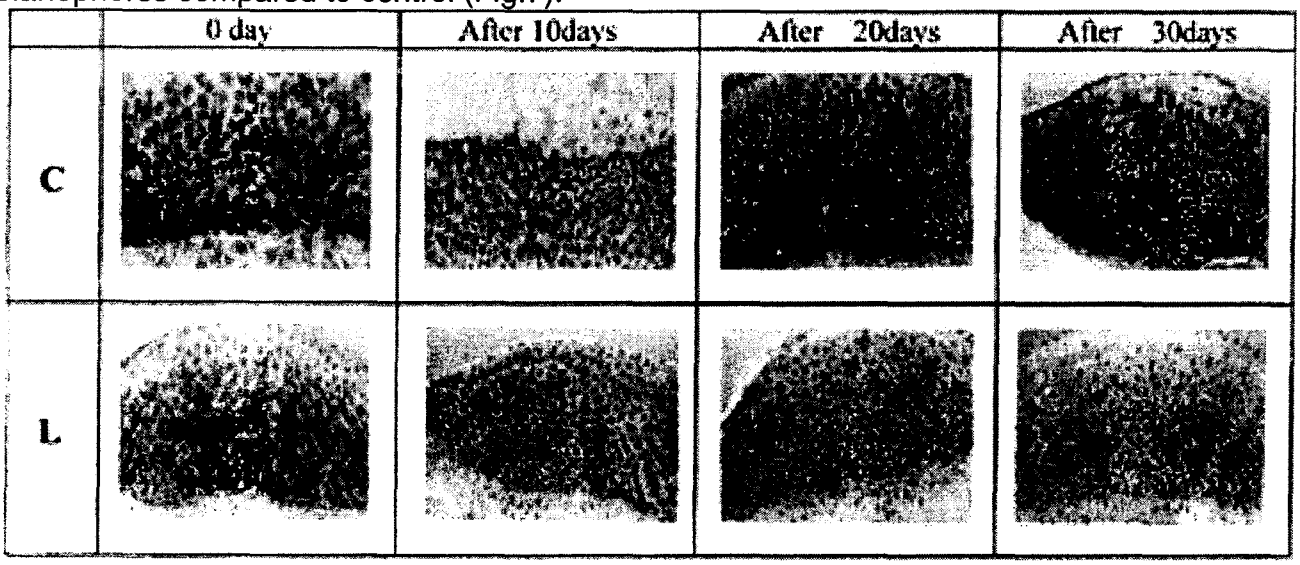


Fig. 6 Effect of Lagenaria leucantha on melanin biosynthesis of S. bikikiensis
A: Control *B: Kojic acid* *C: Lagenaria leucantha*
D: Phellinus linteus E: Arbutin F: 4-hydroxyanisole

Effect of *Lagenaria leucantha* on depigmentation of black gold fish

We confirmed the whitening effect of *Lagenaria leucantha* using black gold fish in vivo. At a concentration of 1 mg/ml, *Lagenaria leucantha* strikingly decreased the size and the number of melanophores compared to control (Fig.7).



Effect of Lagenaria leucantha on depigmentation of black gold fish
Black gold fish were raised in water containing 1mg/ml of Lagenaria leucantha *.*
After 10 days, 20 days and 30 days, the scale from the gold fish was observed using image analyzer.
C: Control L: Lagenaria leucantha

DISCUSSIONS

When skin is exposed to UV, a highly complex cascade of events ensues and culminates in many effects, including increased skin melanin content. These UV -mediated events can be mimicked or enhanced by MSH. In this study, we confirmed that the melanogenesis of cultured B_{16} melanocytes was induced by α -MSH. *Lagenaria leucantha* suppressed the induction of melanogenesis by a -MSH. To examine the inhibitory mechanism of *Lagenaria leucantha*, we evaluated inhibitory activity on tyrosinase and the synthesis of tyrosinase. *Lagenaria leucantha* showed no inhibition of tyrosinase (data was not shown) and weak inhibition on the synthesis of tyrosinase. We have no idea about the mechanism of *Lagenaria leucantha* on the inhibition of melanogenesis but a likely possibility is *Lagenaria leucantha* might inhibit melanogenesis with tyrosinase independent manner. We also confirmed inhibitory effect of *Lagenaria leucantha* on melanin biosynthesis of *S. bikiniensis*. To confirm the inhibitory activity of *Lagenaria leucantha* in vivo, we examined its effect on depigmentation of black gold fish. *Lagenaria leucantha* remarkably reduced the size and density of melanophores. This result suggests that *Lagenaria leucantha* might able to analyze the produced melanin. Although we have not shown the whitening mechanism of *Lagenaria leucantha*, these results suggest that *Lagenaria leucantha* has inhibitory activity against melanogenesis. And *Lagenaria leucantha* is expected that it can be used as a whitening agent in cosmetics.

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