Bioorganic & Medicinal Chemistry 18 (2010) 5241–5247

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

(−)-*N*-Formylanonaine from *Michelia alba* as a human tyrosinase inhibitor and antioxidant

Hui-Min Wang [a,*], Chung-Yi Chen [b], Chun-Yen Chen [c], Mei-Ling Ho [d], Yi-Ting Chou [d], Hou-Chien Chang [e], Chih-Hung Lee [f], Chau-Zen Wang [d], I-Ming Chu [g]

[a] *Department of Fragrance and Cosmetic Science, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC*
[b] *School of Medicine and Health Sciences, Fooyin University, Kaohsiung County 831, Taiwan*
[c] *Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan*
[d] *Department of Physiology, Kaohsiung Medical University, Orthopaedic Research Center, and the Department of Orthopaedics, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan*
[e] *Department of Chemical Engineering, National Chung Hsing University, Taichung 402, Taiwan*
[f] *Department of Dermatology, Kaohsiung Medical University College of Medicine and Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung 807, Taiwan*
[g] *Department of Chemical Engineering, National Tsing Hua University, Hsinchu 300, Taiwan*

ARTICLE INFO

Article history:
Received 16 April 2010
Revised 15 May 2010
Accepted 18 May 2010
Available online 24 May 2010

Keywords:
(−)-*N*-Formylanonaine
Tyrosinase inhibitor
Melanocyte
Molecular docking

ABSTRACT

Tyrosinase is the first and rate limiting enzyme in the synthesis of melanin pigments for coloring hair, skin, and eyes. As reported in this study, a natural product, (−)-*N*-formylanonaine isolated from the leaves of *Michelia alba D.C.* (Magnolianceae), was found to inhibit mushroom tyrosinase with an IC_{50} of 74.3 μM and to have tyrosinase and melanin reducing activities in human epidermal melanocytes without apparent cytotoxicity to human cells, superior to the known tyrosinase inhibitors, such as kojic acid and 1-phenyl-2-thiourea (PTU). Based on homology modeling, the compound binds the active site by coordinating with two Cu^{2+} ions. In addition, the compound had antioxidation activities in tests for scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power, and chelating metal ions. To our knowledge, this is the first study to reveal the bioactivities of (−)-*N*-formylanonaine from this plant species.

1. Introduction

Hyperpigmentations, such as senile lentigo, melasma, freckles, and pigmented acne scars, are of particular concern to women.[1] The treatment usually involves the use of medicines or medicinal cosmetics containing depigmenting agents or skin whitening agents. Tyrosinase (EC 1.14.18.1) is the first and rate limiting enzyme in the biosynthesis of melanin pigments responsible for coloring hair, skin, and eyes.[2,3] Therefore, inhibition of tyrosinase is one of the major strategies to treat hyperpigmentation. Safe and effective tyrosinase inhibitors that act to minimize skin pigmentation abnormalities are desired. However, only a few such as kojic acid and 1-phenyl-2-thiourea (PTU) are used as therapeutic agents, primarily because of various safety concerns and low whitening bioactivity. Tyrosinase catalyzes two distinct significant reactions in melanin synthesis as follows: the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and the oxidation of L-DOPA to dopaquinone followed by further conversion to melanin production.[4,5] In clinical usage, tyrosinase inhibitors are used for treatments of dermatological disorders related to melanin hyper accumulation and are essential in cosmetics for depigmentation.[6–10] Recently, much attention has been drawn to the application of tyrosinase inhibitors to medical treatments or cosmetic businesses.

To prevent diseases, the importance of diet is well recognized.[11–13] Plant species with antioxidant properties can be potentially applied to human health care because of the protective effects in anti-aging and the opposition to various diseases including inflammatory, neurological, and cardiovascular diseases and cancer.[14,15] Antioxidants have the ability to reduce free radical mediated degradation of cells and organs in human beings.[12,16] The generally accepted mechanism is through the scavenging activities of free radical to reduce oxidative stress and prevent the resulting diseases.[17] In addition to wholegrain cereals, oats, legumes, fruits, and vegetables, there are still many other antioxidant natural sources.[18,19]

The genus *Michelia* (Magnoliaceae) consists of approximately 30 species. *Michelia alba* D.C. is a tall tree native to Indonesia and has been used by the native Malays in Indonesia and Malaysia for medicinal purposes.[20] In our continuing investigations of the phytochemical and bioactive compounds of Magnoliaceous plants, (−)-*N*-formylanonaine was chosen as the target compound for human skin whitening and antioxidation. The aim of this study was to

* Corresponding author. Tel.: +886 7 312 1101x2804; fax: +886 7 3210683.
E-mail address: davidw@kmu.edu.tw (H.-M. Wang).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.05.045

evaluate the human tyrosinase inhibition and antioxidation activities of this constituent to see if it can be applied in the cosmetic and food fields. This was the first attempt to demonstrate the essential bioactivities of (−)-*N*-formylanonaine for medical cosmetology and food supplement applications.

2. Results

2.1. Mushroom tyrosinase inhibition

We measured the inhibitory effects of many pure compounds from *M. alba* on in vitro mushroom tyrosinase inhibition assay and found that the (−)-*N*-formylanonaine (structure shown in Fig. 1A) reduced mushroom tyrosinase activity in a dose dependent trend (IC = 74.3 μM), comparable to the IC_{50} of 69.4 μM for kojic acid that is a commonly used human tyrosinase inhibitor in the cosmetic industry (see Table 1). The inhibitory activity of (−)-*N*-formylanonaine may be due to its binding with the enzyme by chelating two copper ions present in the active site as predicted from the computer modeling (Fig. 1B).

2.2. Tyrosinase and melanin reducing activities of (−)-*N*-formylanonaine in human epidermal melanocytes

Next, we examined the effect of (−)-*N*-formylanonaine in reducing tyrosinase activity and melanin content in human melanocytes. As shown in Figure 2A, we found that (−)-*N*-formylanonaine had a similar inhibition effect on human melanocytes as upon the mushroom tyrosinase. When compared to kojic acid and PTU at the same concentration of 100 μM, (−)-*N*-formylanonaine demonstrated higher inhibition on human tyrosinase and melanin content. Furthermore, the melanin contents correlated with the tyrosinase activities in the same dose dependent tendencies upon (−)-*N*-formylanonaine treatment (Fig. 2B). It indicated that the epidermal cellular melanin reductions might be due to the inhibition of human tyrosinase activity. The EC_{50} (the concentration of (−)-*N*-formylanonaine that reduced the melanin content by 50%, relative to the control without test compound) was 90 μM as summarized in Table 2.

2.3. Cytotoxicity of (−)-*N*-formylanonaine in human epidermal melanocytes

3-(4,5-Dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to investigate if the inhibitor adversely induced human melanocyte cell death. The samples were treated with various concentrations from 0.01 μM to 10 μM of (−)-*N*-formylanonaine. Kojic acid and PTU were used as positive controls in the present study. α-Melanocyte stimulating hormone (α-MSH) that is known to stimulate melanogenesis was used at 0.1 μM as the negative control group, and its effect on melanogenesis was compared with that of the testing samples. As shown in Figure 3, cells treated with all testing concentrations of (−)-*N*-formylanonaine exhibited more than 90% of cell viability for up to 48 h, except at an extremely high dose (200 μM). In contrast, cell death was observed at 100 μM of well-known tyrosinase inhibitors kojic acid and PTU, indicating less toxicity of (−)-*N*-formylanonaine. As summarized in Table 2, the EC_{50} and CC_{50} (compound concentration inhibiting cell growth by 50% relative to untreated control) values of (−)-*N*-formylanonaine are 90 μM and 735 μM, respectively, with the selectivity index (SI, CC_{50}/EC_{50}) value of 8.2.

2.4. Morphological changes of human epidermal melanocytes treated with (−)-*N*-formylanonaine

Human skin melanocytes were further used to evaluate the effects of (−)-*N*-formylanonaine. Figure 4A showed the cell morphology of control samples treated with the agents under a phase contrast microscope. Kojic acid and PTU demonstrated melanin reducing abilities, and the color of cells after centrifugation became lighter (data not shown). In contrast, α-MSH stimulated the cellular melanin production, and the cells developed into a darker color.

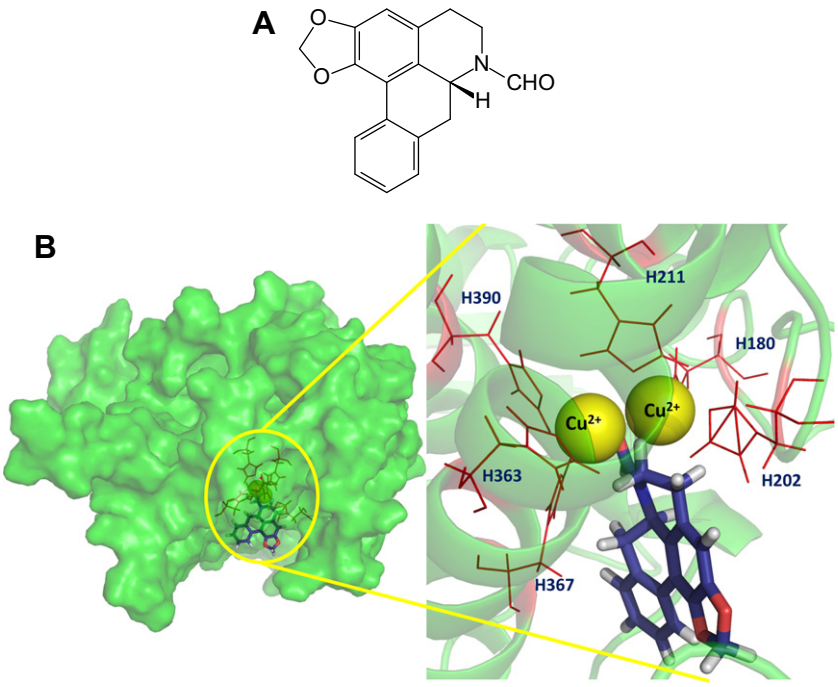

Figure 1. (A) Compound structure of (−)-*N*-formylanonaine from *M. alba*. (B) The proposed binding mode of (−)-*N*-formylanonaine. The active site area is expanded to see it clearly. In the active site, the copper ions are colored in yellow, six key histidine residues in red, main structure of (−)-*N*-formylanonaine in blue, oxygen residues in red and hydrogen residues in white. The docking models were generated using the DS 2.0 modeling program and optimized by energy minimization.

Table 1
IC_{50} values of mushroom tyrosinase inhibition and antioxidant effects of (−)-*N*-formylanonaine

	Mushroom tyrosinase, IC_{50} (μM)	DPPH· scavenging, IC_{50} (μM)	Metal chelating ability, IC_{50} (μM)	Reducing power (at 100 μM, OD_{700})
(−)-*N*-Formylanonaine	74.3	121.4	262.1	0.56
Kojic acid[a]	69.4	—	—	—
Vitamin C[b]	—	52.1	—	—
EDTA[c]	—	—	0.1	—
BHA[d]	—	—	—	1.28

Data were expressed as a mean value of at least three independent experiments. (−) is no testing.
[a] Kojic acid was used as a positive control of mushroom tyrosinase assay.
[b] Vitamin C was used as a positive control on DPPH assay.
[c] EDTA was used as a positive control on metal chelating ability.
[d] BHA was used as a positive control on reducing power at 100 μM.

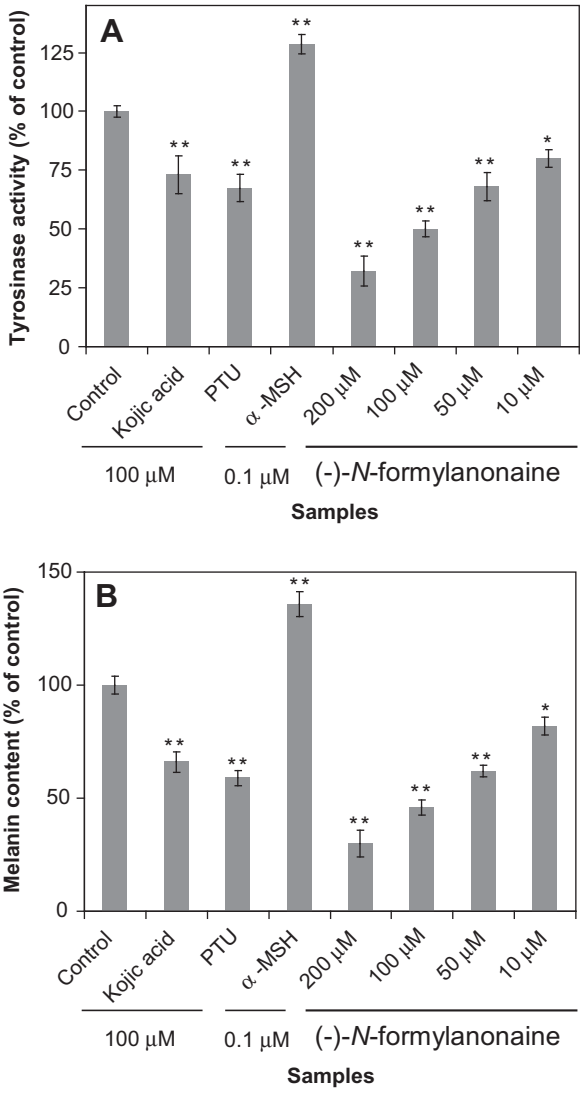

Figure 2. Human tyrosinase (A) and melanin (B) inhibition on melanocytes in the presence of various concentrations of (−)-*N*-formylanonaine. Kojic acid and PTU were used as melanin inhibitors at 100 μM, and α-MSH was a melanin stimulator at 0.1 μM. Operation procedures are in Section 4. (* <0.01 and ** <0.001).

We found that after exposure to (−)-*N*-formylanonaine, the morphology of the majority of human melanocytes changed from original dendritic morphology to a bipolar and fibroblast cell type in dose dependent manners (Fig. 4B). When exposed to higher doses, the melanocyte cells had little dendritic morphology. Images of melanocytes separately treated with 10 μM and 200 μM of (−)-*N*-formylanonaine were taken at five intervals from 12 h to 60 h (Fig. 4C and D). We also discovered that the dendritic morphology at both concentrations was reduced, and the phenomenon was more substantial at 200 μM when compared to 10 μM. At the same time, melanin productions apparently decreased, which implied that loss of dendritic morphology may be related to the reduction of melanin production abilities. It is noteworthy that no apparent cell death was observed after the first 48 h of (−)-*N*-formylanonaine treatments. Thus, the morphological change was not the primary lethal event in human melanocytes.

Table 2
CC_{50}, and EC_{50} of the (−)-*N*-formylanonaine on cell-based assays

	CC_{50} (μM)	EC_{50} (μM)	SI
Melanocytes	735	90	8.2
Keratinocytes	355	—	—
Fibroblasts	325	—	—

Data were expressed as a mean value of at least three independent experiments. (−) is no testing.

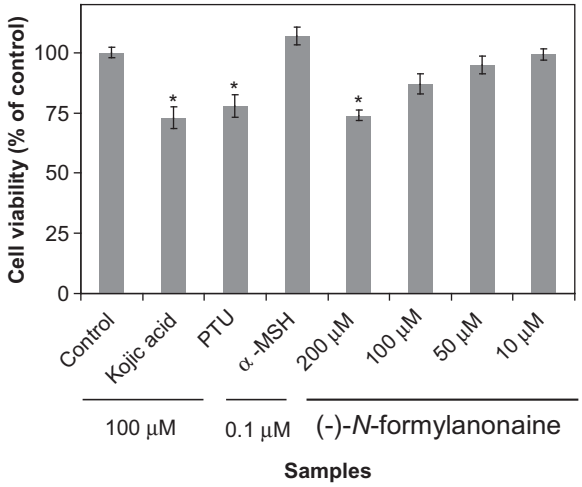

Figure 3. HEMn-MP cell viability with various doses of (−)-*N*-formylanonaine. Kojic acid and PTU were used as melanin inhibitors at 100 μM, and α-MSH was a melanin stimulator at 0.1 μM. Operation procedures are in Section 4. (* <0.01).

2.5. Cytotoxicity of (−)-*N*-formylanonaine in human keratinocytes and fibroblasts

As melanogenic regulatory compounds are being developed, potential safety measures need to be taken into consideration, such as tests for sensitivity, allergies or toxicity. While (−)-*N*-formylanonaine has potential cosmetic or therapeutic uses in human beings, the cytotoxic properties in other human skin cells (skin epidermal keratinocytes and dermal fibroblasts) were evaluated with a more physiologically relevant method in addition to the in vitro screening in human primary epidermal melanocytes. Figure 5 indicates the cell viability of human keratinocytes and fibroblasts treated with various concentrations of (−)-*N*-formylanonaine for 48 h. Treatment with (−)-*N*-formylanonaine at a low dose (10 μM) did not yield significant cytotoxicity in keratinocytes or fibroblasts. After treating skin fibroblasts with 100 μM of (−)-*N*-formylanonaine, cell viability was more than 70%, and after being treated with 200 μM of (−)-*N*-formylanonaine for 48 h, more than half of the fibroblasts survived. The CC_{50} values of human keratinocytes and

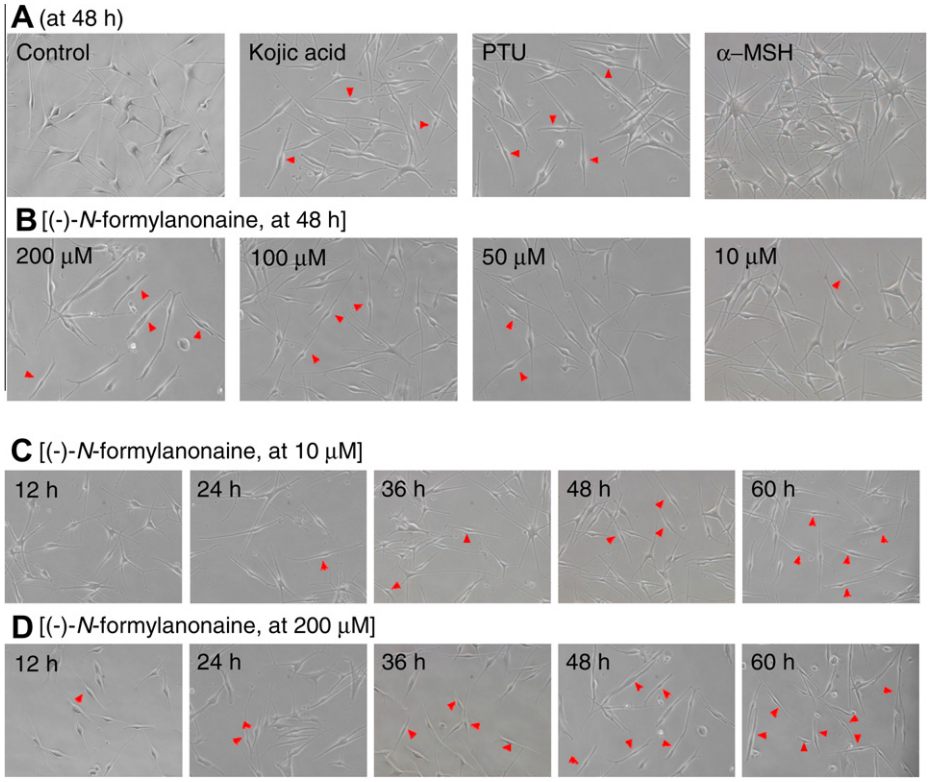

Figure 4. After 48 h of treatment, the images of HEMn-MP were observed under a phase contrast bright field microscope. (A) Images of control groups including kojic acid and PTU as melanin inhibitors at 100 μM and α-MSH as a melanin stimulator at 0.1 μM. (B) Various concentrations of (−)-*N*-formylanonaine. (C and D) Time dependent factors of the morphology of HEMn-MP cells after treated with 10 μM and 200 μM of (−)-*N*-formylanonaine. Magnification is 200× without staining. Red arrows indicate the HEMn-MP cells with bipolar shape morphology.

fibroblasts on (−)-*N*-formylanonaine are approximately 355 μM and 325 μM, respectively (Table 2).

2.6. Anti-oxidation activities

In a DPPH free radical scavenging system, antioxidants act to inhibit oxidation products. Therefore, the scavenging of DPPH radicals was used in this investigation. In this assay, antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. The IC_{50} for (−)-*N*-formylanonaine to inhibit DPPH is 121.4 μM (Table 1). (−)-*N*-formylanonaine had middle to high inhibitory effects in the DPPH assay when compared to vitamin C that has IC_{50} of 52.1 μM (Table 1).

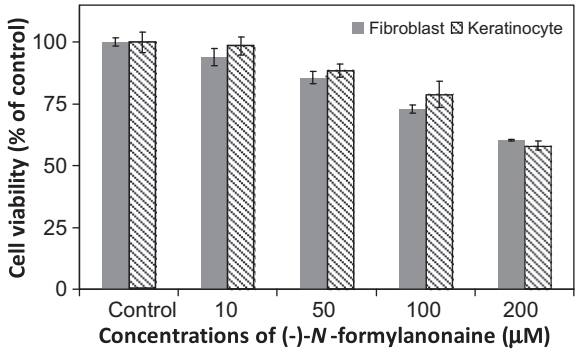

Figure 5. Human keratinocytes and fibroblasts cell viabilities with (−)-*N*-formylanonaine at various concentrations for 48 h. The solid gray bar represents fibroblast cells, and the striped bar represents keratinocyte cells.

The ferrous ion chelating activities of (−)-*N*-formylanonaine are shown in Table 1. Ferrozine quantitatively formed complexes with Fe^{2+}. With the existence of chelating agents, such as (−)-*N*-formylanonaine, the complex was disrupted resulting in a lightening of the red color. The IC_{50} value of (−)-*N*-formylanonaine was 261.2 μM with a minor level of Fe^{2+} scavenging effect, whereas ethylene diamine tetra-acetic acid (EDTA) presented a strong scavenging ability with IC_{50} of 0.1 μM (Table 1).

In this assay, the color of the testing solutions altered from yellow to different shades between green and blue depending upon the reducing power of these antioxidants. The presence of (−)-*N*-formylanonaine similar to the antioxidant substances in the antioxidant samples induced the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Table 1 summarized the reducing power of (−)-*N*-formylanonaine at 100 μM (OD_{700} = 0.56), a moderate level of potential compared to 3-*tert*-butyl-4-hydroxyanisole (BHA) at the same dose (OD_{700} = 1.28).

3. Discussion

This work is a novel biochemical characterization of (−)-*N*-formylanonaine properties because there were no reports in previous cosmetics or food applications using this natural product. Through the in vitro mushroom tyrosinase inhibitor screening, we targeted (−)-*N*-formylanonaine from isolated compounds of *M. alba*. Our studies thus focused on melanin inhibition in human epidermal melanocytes by the compound. In human skin, melanosomes are released from melanocyte dendrites and taken directly up by keratinocytes through endocytosis or phagocytosis.[21] In previous keratinocyte and melanocyte co-culture studies, we found that as melanocytes lose their characteristic dendritic structures and adopt fibroblast-like bipolar forms, the cell–cell contact

between melanocytes and keratinocytes is considerably reduced resulting in a reduction in pigment transfer.[22] We discovered that tyrosinase activity and melanin levels were significantly decreased by the addition of (−)-*N*-formylanonaine in a dose dependent manner in human epidermal melanocytes. The results indicate that it suppresses tyrosinase activity and total melanin content without having adverse affects. Furthermore, several keratinocytes derived factors also enhance the dendricity of isolated melanocytes.[23] However, little was known concerning the relationships between the morphology and the melanin content or tyrosinase activities of the melanocytes. Our study suggests that there may be some connections between the dendritic morphology changes and physiological properties of melanocytes. These potential relationships should be further investigated to understand their physiological significances.

(−)-*N*-Formylanonaine not only inhibits tyrosinase activity in melanocyte cells, but also exhibits human skin antioxidation activities, potentially useful in both medical cosmetology as well as food supplementation. The antioxidation assays show that (−)-*N*-formylanonaine has moderate antioxidant abilities in DPPH scavenging activity, reducing power and metal chelating. The formation of free radicals has been associated with human aging, and consumers tend to prefer fresh, non-artificial natural products like (−)-*N*-formylanonaine as antioxidant supplements to scavenge radicals and prevent deleterious effects of free radicals in skin care, foods, and biological systems. Excessive formation and accumulation of free radicals accelerate the oxidation of lipids in cosmetics and foods, thereby decreasing product quality and consumer acceptance.[24] The antioxidant property of (−)-*N*-formylanonaine may be directly influenced by its redox properties, which may have an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. Therefore, it may be influenced by the supply of hydrogen combining with radicals because (−)-*N*-formylanonaine produced a stable radical to cease the radical chain reaction.[25] It is also possible that (−)-*N*-formylanonaine is able to join with the radical ions required for the radical chain reaction followed by the termination of the chain. We therefore proposed that (−)-*N*-formylanonaine is a potent and novel tyrosinase inhibitor serving as a basis in dermatological drug development.

4. Materials and methods

4.1. Reagents and materials

Mushroom tyrosinase, vitamin C, dimethyl sulfoxide (DMSO), kojic acid, PTU, α-MSH, L-tyrosine, L-DOPA, DPPH, EDTA, BHA, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid, $FeCl_3$, $FeCl_2{\cdot}4H_2O$, and MTT were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD). All buffers and other reagents were of the highest purity and commercially available.

4.2. Preparation of (−)-*N*-formylanonaine

(−)-*N*-Formylanonaine (Fig. 1, purity >90%) was isolated from the leaves of *M. alba* as described previously.[26,27] Briefly, the air-dried leaves (6.0 kg) were extracted with methanol (MeOH; 70 L with six times) at room temperature, and the extract (367.8 g) was obtained upon concentration under reduced pressure. The extract was suspended in H_2O (1 L) and was partitioned with chloroform ($CHCl_3$; 2 L with six times) to give fractions soluble in $CHCl_3$ (154.3 g) and H_2O (144.1 g). The $CHCl_3$ soluble fraction was chromatographed over a silica gel (70–230 mesh) using *n*-hexane/ethyl acetate (EtOAc)/$CHCl_3$/MeOH mixtures as an eluent to produce six fractions. A part of the sixth fraction (25.0 g) was subjected to silica gel chromatography by eluting with $CHCl_3$/MeOH (60:1) and then enriched with MeOH to produce five fractions (6-1 to 6-5). Fraction 6-1 (4.23 g) was subjected to silica gel chromatography by eluting with $CHCl_3$/MeOH (60:1) and then enriched gradually with MeOH to produce three fractions (6-1-1 to 6-1-3). Fraction 6-1-3 (1.28 g) was further purified by passage over another silica gel column using $CHCl_3$/MeOH mixtures to yield (−)-*N*-formylanonaine (18.0 mg). The ^{1}H NMR spectral data were consistent with the structure (^{1}H NMR (400 MHz, $CDCl_3$): *E* form: δ 2.74 (1H, dd, J = 10.8, 2.4 Hz H-4α), 2.84 (1H, dd, J = 14.0, 4.4 Hz, H-7β), 2.89 (1H, m, H-4β), 3.24 (1H, dd, J = 14.0, 4.4 Hz H-7α), 3.41 (1H, td, J = 12.0, 2.4 Hz, H-5β), 3.82 (1H, ddd, J = 12.8, 4.8, 3.6 Hz, H-5α), 5.06 (1H, dd, J = 13.6, 4.4 Hz, H-6a), 5.99 and 6.11 (each 1H, d, J = 1.2 Hz, –OCH_2O–), 6.59 (1H, s, H-3), 7.25–7.35 (3H, m, H-8,9,10), 8.10 (1H, d, J = 7.6 Hz, H-11), 8.27 (1H, s, CHO)).

4.3. Bioassay methods

4.3.1. Assay on mushroom tyrosinase activity

Tyrosinase activity inhibition was determined spectrophotometrically according to the method previously described with minor modifications.[28,29] Assays were conducted in a 96-well microplate with an optics plate reader used to determine the absorbance at 490 nm (Molecular Devices; reference: 655 nm). Kojic acid was used as a positive control for the tyrosinase inhibitory assay. The testing substance was dissolved in aqueous DMSO and incubated with L-tyrosine (2.5 mg/mL) in a 50 mM phosphate buffer (pH 6.8). All samples were dissolved in DMSO, which did not affect tyrosinase activity when DMSO was less than 1% of the total volume. Then 25 U/mL of mushroom tyrosinase in the same buffer was added, and the mixture was incubated at 37 °C for 30 min (Table 1). Tyrosinase inhibitory activity was determined by the following equation:

$$\%\ \text{inhibition} = 100\% \times \{[(A - B) - (C - D)]/(A - B)\} \quad (1)$$

In this equation, A is the optical density (OD_{490}) without testing substance, B is the OD_{490} without testing substance but with tyrosinase, C is the OD_{490} with testing substance and D is the OD_{490} with testing substance but without tyrosinase. The IC_{50} value of the inhibitor was determined by fitting the initial rates versus inhibitor concentrations using the following equation:

$$E(I) = E(0) \times \{1 - [I/(I + IC_{50})]\} \quad (2)$$

In this equation, $E(I)$ is the enzyme activity with inhibitor concentration I, $E(0)$ is the enzyme activity without inhibitor, I is the inhibitor concentration and IC_{50} is the concentration of inhibitor that caused half of a reaction rate.

4.3.2. Molecular docking of human tyrosinase

Since no three-dimensional structure for human tyrosinase is currently available, a theoretical homology model was retrieved for the present docking study. To construct a human tyrosinase model with an active site in the likely ligand bound protein conformation, a homology model was first simulated by computer docking with the substrate L-DOPA and two copper ions using molecular energy minimization and dynamic simulation.[30] Briefly, the crystal structure of *Octopus dofleini* hemocyanin (1JS8) was downloaded from Protein Data Bank (www.pdb.com). The active site was docked with inhibitors using the dock suite of Accelrys Discovery Studio 2.0 software (Accelrys, Inc.). All crystallographic water molecules, solvent molecules and ions were removed from the protein structure. The binding site was defined with the following options: site opening = 5 Å and grid resolution = 0.5 Å. Docking was performed with default values selected for the energy grid forcefield, and 'Minimizer' was selected for the minimization

algorithm. The preferable orientation of the protein with compound was then presented. To present molecular docking results, PyMOL software was used to show the homology structure of human tyrosinase with (−)-*N*-formylanonaine.

4.3.3. Human cell culture

Human keratinocytes were grown from foreskin primary culture that was derived from Chung-Ho Memorial Hospital, Kaohsiung Medical University, Taiwan. They were kind gifts from Dr. Chih-Hung Lee. Human keratinocytes were cultured in Keratinocyte-SFM (10724; GIBCO™) supplemented with Bovine Pituitary Extract (cat. #13028-014) and EGF Human Recombinant (cat.#10450-013). Neonatal foreskin primary human epidermal melanocytes were purchased from Cascade Biologics™ and cultured in Medium 254 (M-254-500; Cascade Biologics™) supplemented with human melanocyte growth supplement (HMGS; cat.# S-002-5). The primary cultures of human skin fibroblasts were complimentary gifts from professor Yau-Huei Wei. All cell types were incubated at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. To study melanin biogenesis, human melanocytes were incubated in 24-well plates at a density of 1×10^5 cells per well. All cells were treated with various concentrations of the inhibitor.

4.3.4. Cytotoxicity assay – MTT assay

The MTT assay was used to determine cell viability and proliferation.[30,31] All of the cell lines were seeded in 24-well microplates. After seeding cells for 24 h, various compounds with concentrations ranging from 10 μM to 200 μM were added. Within 48 h of compound treatments, images of HEMn-MP and human skin fibroblasts were taken at suitable time intervals (Multiskan Ascent V1.24, Ascent Software Version 2.6). MTT solution (5 mg/mL and dissolved in phosphate buffered saline; PBS) was diluted 1:10 in culture medium and added to a culture dish followed by an incubation at 37 °C. After 2 h of MTT treatment, the media was removed and each precipitate in a specific dish was dissolved in 100 μL of DMSO to dissolve the purple formazan crystals. After the dishes were gently shaken for 20 min in the dark to ensure maximal dissolution of formazan crystals, the optical density (OD) values of the supernatant were measured at 595 nm. All experiments were repeated at least three times. In consideration of the possible antiproliferative effects of DMSO, a maximal amount (0.5%) of DMSO was added to culture and used as positive controls. DMSO at this amount was found not to affect the growth of the skin cells.

4.3.5. Assay on cellular tyrosinase activity

In human skin melanocytes, tyrosinase activity was measured by the conversion of L-tyrosine and oxidation of L-DOPA to dopaquinone. The cellular tyrosinase inhibition ability of (−)-*N*-formylanonaine was based on a previously described method with some modifications.[32] Human melanocytes (10^5 per well) were placed in 24-well plates in 500 μL of medium containing various concentrations of testing samples and were incubated for 2 d. The treated cells were washed with PBS and lysed with 0.5% Triton X-100/PBS. The lysates were mixed by vibration with 10 μL of 10 mM L-tyrosine and 10 mM L-DOPA in 0.1 M phosphate buffer (pH 6.8). After incubation at 37 °C for 3 h, the absorbance at 490 nm was measured on a spectrophotometer.

4.3.6. Melanin quantification assay

Briefly, we followed a previously described method with some minor modifications.[33] Cell pellets were dissolved in 1.0 N NaOH containing 10% DMSO and heated at 80 °C for 1 h. Suspensions were then clarified by centrifugation for 10 min at 10,000 *g*. The amount of melanin was determined spectrophotometrically based on the absorbance at 475 nm.

4.3.7. Determination of DPPH radical scavenging capacity

Most cosmetics and food compounds have free radical scavenging abilities. The antioxidant activity of the target compound was measured in terms of hydrogen donating or radical scavenging ability using the modified DPPH method.[33,34] Different concentrations of the samples were added to 0.2 mL of a DPPH (60 μM) solution. When DPPH reacts with an antioxidant compound that donates hydrogen, it is reduced resulting in a decrease in the absorbance at 520 nm. The absorbance was recorded at 5 min intervals for 30 min using a UV visible spectrophotometer and was evaluated at the end point (30 min). Vitamin C was used as a positive control. The percentages of remaining DPPH were plotted against the sample to obtain the amount of antioxidant required to reduce the initial concentration of DPPH. Scavenging activity (%) was determined with the following equation:

$$100 \times (OD_{control} - OD_{sample})/OD_{control} \quad (3)$$

4.3.8. Metal chelating activity

The ferrous ion chelating potential of chlorophyll was investigated according to a previously described method.[35] Briefly, various testing concentrations of samples dissolved in DMSO were added to a solution of 2 mM $FeCl_2 \cdot 4H_2O$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and the mixture was vigorously shaken and left standing at room temperature for 10 min. The absorbance of the mixture was then read at 560 nm against a blank. EDTA was used as a positive control, and the formula for calculation of the chelating activity was similar to Eq. (3).

4.3.9. Reducing power assay

The reducing power of (−)-*N*-formylanonaine was determined according to a previously described method.[36] Various concentrations of testing samples in 0.063 mL of methyl alcohol were mixed with 0.1 mL of 0.2 M sodium phosphate buffer (pH 6.8) and 2.5 μL of 20% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50 °C for 20 min, and 0.16 mL of trichloroacetic acid (10%) was then added to the mixture that was then centrifuged for 10 min at 3000*g*. The upper layer of the solution (75 μL) was mixed with distilled water (25 μL) and 2% $FeCl_3$ (25 μL), and the absorbance was measured with a 96-well plate spectrophotometer at 650 nm. BHA was used as a positive control. A higher absorbance demonstrates a higher reductive capability.

4.4. Statistical analysis

Results are presented as a mean value of the data obtained from at least triplicate experiments. Student's *t*-test was used to determine the level of significance.

Acknowledgments

This investigation was financially supported by the National Science Council of Taiwan, ROC under the Grant Numbers of NSC97-2221-E-037-002-, NSC98-2221-E-037-005-, NSC-97-2320-B-242-002-MY3 and NSC 97-2321-B-037-001-MY2; Ministry of Economic Affairs, 98-EC-17-A-10-S2-0066; and by the Kaohsiung Medical University under Grant Number Q097002. The authors would like to thank Xiu-Wei Kuo, Ya-Ling Yeh and Yung-Hsiu Chen for their assistance. The authors would also like thank Dr. Yau-Huei Wei and Min-Yii Julie Wang for the English editing.

References and notes

1. Tripathi, R. K.; Hearing, V. J.; Urabe, K.; Aroca, P.; Spritz, R. A. *J. Biol. Chem.* **1992**, *267*, 23707.
2. Hirobe, T. *Pigment Cell Res.* **2005**, *18*, 2.
3. Kim, J. H.; Baek, S. H.; Kim, D. H.; Choi, T. Y.; Yoon, T. J.; Hwang, J. S.; Kim, M. R.; Kwon, H. J.; Lee, C. H. *J. Invest. Dermatol.* **2008**, *128*, 1227.
4. Solano, F.; Briganti, S.; Picardo, M.; Ghanem, G. *Pigment Cell Res.* **2006**, *19*, 550.
5. Marles, L. K.; Peters, E. M.; Tobin, D. J.; Hibberts, N. A.; Schallreuter, K. U. *Exp. Dermatol.* **2003**, *12*, 61.
6. Shiino, M.; Watanabe, Y.; Umezawa, K. *Bioorg. Med. Chem.* **2001**, *9*, 1233.
7. Gillbro, J. M.; Marles, L. K.; Hibberts, N. A.; Schallreuter, K. U. *J. Invest. Dermatol.* **2004**, *123*, 346.
8. Spencer, J. D.; Chavan, B.; Marles, L. K.; Kauser, S.; Rokos, H.; Schallreuter, K. U. *J. Endocrinol.* **2005**, *187*, 293.
9. Schallreuter, K. U.; Hasse, S.; Rokos, H.; Chavan, B.; Shalbaf, M.; Spencer, J. D.; Wood, J. M. *Exp. Dermatol.* **2009**, *18*, 680.
10. Wood, J. M.; Decker, H.; Hartmann, H.; Chavan, B.; Rokos, H.; Spencer, J. D.; Hasse, S.; Thornton, M. J.; Shalbaf, M.; Paus, R.; Schallreuter, K. U. *FASEB J.* **2009**, *23*, 2065.
11. Sur, P.; Chaudhuri, T.; Vedasiromoni, J. R.; Gomes, A.; Ganguly, D. K. *Phytother. Res.* **2001**, *15*, 174.
12. Wongkham, S.; Laupattarakasaem, P.; Pienthaweechai, K.; Areejitranusorn, P.; Wongkham, C.; Techanitiswad, T. *Phytother. Res.* **2001**, *15*, 119.
13. Yao, S.; Tan, H.; Zhang, H.; Su, X.; Wei, W. *Biotechnol. Prog.* **1998**, *14*, 639.
14. Scalbert, A.; Johnson, I. T.; Saltmarsh, M. *Am. J. Clin. Nutr.* **2005**, *81*, 215S.
15. Lu, Y.; Foo, L. Y. *Food Chem.* **1997**, *59*, 187.
16. Jin, D.; Hakamata, H.; Takahashi, K.; Kotani, A.; Kusu, F. *Biomed. Chromatogr.* **2004**, *18*, 662.
17. Huang, Y. L.; Chen, C. C.; Chen, Y. J.; Huang, R. L.; Shieh, B. J. *J. Nat. Prod.* **2001**, *64*, 903.
18. Nihal, M.; Ahmad, N.; Mukhtar, H.; Wood, G. S. *Int. J. Cancer* **2005**, *114*, 513.
19. Karakaya, S.; Kavas, A. *J. Sci. Food Agric.* **1999**, *79*, 237.
20. Bentley, K. W. *Nat. Prod. Rep.* **1999**, *16*, 367.
21. Lin, H. C.; Shieh, B. H.; Lu, M. H.; Chen, J. Y.; Chang, L. T.; Chao, C. F. *Pigment Cell Melanoma Res.* **2008**, *21*, 559.
22. Regnier, M.; Tremblaye, C.; Schmidt, R. *Pigment Cell Res.* **2005**, *18*, 389.
23. Gordon, P. R.; Mansur, C. P.; Gilchrest, B. A. *J. Invest. Dermatol.* **1989**, *92*, 565.
24. Gulcin, I.; Elmastas, M.; Aboul-Enein, H. Y. *Phytother. Res.* **2007**, *21*, 354.
25. Wang, S. Y.; Ballington, J. R. *LWT-Food Sci. Technol.* **2007**, *40*, 1352.
26. Chen, C. Y.; Huang, L. Y.; Chen, L. J.; Lo, W. L.; Kuo, S. Y.; Wang, Y. D.; Kuo, S. H.; Hsieh, T. *J. Chem. Nat. Compd.* **2008**, *44*, 137.
27. Wang, H. M.; Lo, W. L.; Huang, L. Y.; Wang, Y. D.; Chen, C. Y. *Nat. Prod. Res.* **2010**, *24*, 398.
28. Likhitwitayawuid, K.; Sritularak, B. *J. Nat. Prod.* **2001**, *64*, 1457.
29. Chen, C. Y.; Kuo, P. L.; Chen, Y. H.; Huang, J. C.; Ho, M. L.; Lin, R. J.; Chang, J. S.; Wang, H. M. *J. Taiwan Inst. Chem. Eng.* **2010**, *41*, 129.
30. Lin, Y. P.; Hsu, F. L.; Chen, C. S.; Chern, J. W.; Lee, M. H. *Phytochemistry* **2007**, *68*, 1189.
31. Chen, B. H.; Wu, P. Y.; Chen, K. M.; Fu, T. F.; Wang, H. M.; Chen, C. Y. *J. Nat. Prod.* **2009**, 950.
32. Yoshimura, K.; Tsukamoto, K.; Okazaki, M.; Virador, V. M.; Lei, T. C.; Suzuki, Y.; Uchida, G.; Kitano, Y.; Harii, K. *J. Dermatol. Sci.* **2001**, *27*, S68.
33. Wang, K. J.; Zhang, Y. J.; Yang, C. R. *J. Ethnopharmacol.* **2005**, *99*, 259.
34. Chen, C. Y.; Wu, P. Y.; Huang, T. S.; Lin, C. W.; Li, Y. C.; Chou, R. H.; Chang, H. W.; Wang, H. M. *Curr. Nutr. Food Sci.* **2009**, *5*, 172.
35. Decker, E. A.; Welch, B. *J. Agric. Food Chem.* **1990**, *38*, 674.
36. Elmastas, M.; Turkekul, I.; Ozturk, L.; Gulcin, I.; Isildak, O.; Aboul-Enein, H. Y. *Comb. Chem. High Throughput Screening* **2006**, *9*, 443.