Original Article

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Hispidin and related herbal compounds from *Alpinia zerumbet* inhibit both PAK1-dependent melanogenesis in melanocytes and reactive oxygen species (ROS) production in adipocytes

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Summary **Recently several compounds from Okinawa plants including *Alpinia zerumbet* (alpinia) were shown to inhibit directly the oncogenic/ageing kinase PAK1 (p21-activated kinase 1). Furthermore, it was recently revealed that both PAK1 and PAK4 (p21-activated kinase 4) are equally essential for the melanogenesis in melanoma cells. Thus, in this study, we tested if several alpinia compounds inhibit the melanogenesis in melanoma (B16F10) cells, as well as the PAK1-dependent up-regulation of both reactive oxygen species (ROS) and nitric oxide (NO) in cultured adipocytes (3T3-L1) without any cytotoxicity. The effect of alpinia compounds on the melanogenesis was measured by both the melanin content and intracellular tyrosinase activity in melanoma cells treated with 3-isobutyl-1-methylxanthine (IBMX), a melanogenesis stimulating hormone. We found that (1E,3E,5E)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD), 5,6-dehydrokawain (DK), labdadiene, hispidin and dihydro-5,6-dehydrokawain (DDK) at 50 μg/mL reduced the melanin content by 63-79%. The MTD, DK and hispidin, at 50 μg/mL, inhibited tyrosinase activity by 70-83% in melanoma cells. Among these compounds, labdadiene, MTD, (E)-2,2,3,3-Tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1H-quinolizine (TMOQ) and hispidin strongly inhibited the ROS production. Hispidin, labdadiene and MTD at 20 μg/mL inhibited NO production by over 70%. These findings altogether suggest that some of these alpinia compounds could be potentially useful for the prevention or treatment of hyper-pigmentation and obesity.**

Keywords: *Alpinia zerumbet*, ROS, melanogenesis, MTD, labdadiene, hispidin, PAK1

1. Introduction

Alpinia zerumbet (alpinia) belongs to the Zingiberaceae, which widely distributes in South-East Asia areas such as Taiwan and Okinawa Islands. As a traditional folk medicine, it has been used for the treatment of flu and pain (*1*). Several years ago, extract of alpinia seeds was found to have a potent hypo-lipidemic effect *in vivo* (*2*). More recently, a variety of pharmacological activities have been reported on isolated alpinia compounds. For instance, both 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK) inhibit the major oncogenic kinase PAK1 (RAC/CDC42-activated kinase 1) (*3*) and block the platelets aggregation which depends on PAK1 (*4*). Furthermore, DK and DDK also inhibit PAK1-dependent viral infection by interfering with both HIV-integrase and neuraminidase (*5,6*). Beside, labdadiene has an anti-atherosclerosis activity and inhibits lipid peroxidation, cyclo-oxygenases and cancer growth (*7,8*). Hispidin, a metabolite of DK, also inhibits a few oncogenic kinases including protein kinase C (PKC)

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and PAK1, and possesses the anti-obesity and anti-cancer activities (*9,10*).

Melanin pigments, eumelanin and pheomelanin, are responsible for dark skin color (*11*), and protect the skin from UV-induced injury (*12*). However, the over-production of melanin in the skin leads to pigmentary disorders including dryness of the skin, irregular pigmentation-freckles, lentigines, wrinkling, and inelasticity (*13*). Melanin is synthesized in the melanocytes *via* an enzymatic cascade with tyrosinase as a key enzyme, and expression of tyrosinase gene depends on a few oncogenic/melanogenic transcription factors such as beta-catenin and microphthalmia-associated transcription factor (MITF), that are inactivated by a variety of herbal polyphenols such as tiliroside from raspberry and CAPE (caffeic acid phenethyl ester) from propolis (*14,15*). Interestingly, beta-catenin is among the direct substrates of the oncogenic kinases such as PAK1 and PAK4 (CDC42-activated kinase 4).

Very recently it was revealed that the melanogenesis in skin cells depends on two distinct members of PAK family, the oncogenic kinases PAK1 and PAK4 (*16*, Be Tu *et al*., manuscript submitted). Each appears to contribute independently by around 50%. Furthermore, we recently demonstrated that a variety of compounds from Okinawa plants, including hispidin, DK and DDK from alpinia, directly inhibit PAK1 (*3*). Moreover, we recently found that alpinia extract significantly extends the healthy lifespan of *Caenorhabditis elegans* (*17*). The phenotype of this worm treated with alpinia extract is very similar to that of PAK1-deficient mutant (RB689) of this worm which lives longer than the wild-type by more than 50% (*18*).

Thus, in this study, we tested if several compounds from alpinia rhizome or seeds such as hispidin, DK, DDK, labdadiene,(1E,3E,5E)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD) and (E)-2,2,3,3-Tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1H-quinolizine (TMOQ) inhibit the melanogenesis as well as both reactive oxygen species (ROS) and nitric oxide (NO) production in cultured 3T3-L1 adipocytes which are associated with obesity and known to be PAK1-dependent, as a PAK1-blocker called CAPE inhibits the melanogenesis as well as obesity and ROS/NO production (*15,19-21*). The outcome of our present study suggests that some of these alpinia compounds would be useful for treatment of both PAK1/PAK4-dependent hyper-pigmentation and PAK1-dependent obesity.

2. Materials and Methods

2.1. *Plant materials and reagents*

The rhizomes and seeds of alpinia were collected from Ryukyus University campus in Okinawa, Japan.

Insulin, Oil Red O and nitro blue tetrazolium (NBT) tablets were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). 3T3-L1 cells and B16F10 melanoma cells were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). Calf serum (CS) was obtained from Thermo Scientific (Old Highway, Tauranga, New Zealand). Dulbecco's modified Eagle medium (DMEM), dexamethasone, 3-isobutyl-1-methyl xanthine (IBMX), sodium nitrite, fetal bovine serum (FBS), triton-X, and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All reagents were of the highest grade available.

2.2. *Preparation of DK, DDK and hispidin from alpinia*

DK and DDK were isolated from the alpinia rhizomes as described previously (*6*) (Figures 1a and 1b).The conversion of DK to hispidin by rat liver microsomal protein was performed according to Tang *et al*. (*22*) with a few minor modifications (Figure 2).

2.3. *Preparation of labdadiene, MTD and TMOQ from alpinia*

Labdadiene was isolated from the alpinia seeds. The seeds (500 g) were extracted with 1 L of ethanol by maceration at room temperature for 2 days. After filtration, the filtrate was evaporated to dryness to give 3.26 g of dark syrupy extract. The extract was suspended in distilled water (300 mL) and partitioned with hexane (300 mL) and ethyl acetate (EtOAc) (300 mL).The EtOAc extract (1.02 g) was subjected to glass chromatography column containing silica gel (Silica gel 60N, particle size 63-120 μm, 70-230 mesh ASTM) and eluted with hexane: acetone (0-100%) to afford three fractions, fraction 1 was further used thin layer chromatography (TLC). The solvent used for isolate labdadiene (hexane: acetone, 9: 1, v/v). The TLC pre-coated silicagel (Merk-60 254, 0.25 mm thick) plate were developed using a Camag twin-through glass tank which was pre-saturated with the mobile phase for 45 min and each plate was developed to a height about 10 cm. After development plate was removed and dried and sports were visualized in UV light. Then, preparative thin layer chromatography (PTLC) was used to collect labdadiene, 50 mg from fraction 1 were dissolved in acetone and the plate was developed in hexane: acetone (9:1, v/v) with total volumes of 200 mL. Four bands were marked (under 254 nm) and then scraped from the plate. The scraping from each band was extracted (2-3 times), by stirring with EtOAc. Band 1 (major broad band) when extracted with EtOAc, gave 21.2 mg of steroid. Band 2 gave 5.6 mg, band 3 gave 6.0 mg and band 4 gave 18.2 mg. ^{1}H-NMR spectra were obtained in methanol-d_4 with a ULTRASHIELD^{TM} PLUS 500 MHz (Bruker Biospin, Germany). Chemical shifts (δ) were obtained

using standard pulse sequences on TopSpin 2.1 program Version 2.1.0 and reported in part per million (ppm). The signals are expressed as singlet (*s*), doublet (*d*), triplet (*t*), quarter (*q*) and multiplet (*m*). Coupling constants (*J*) are reported in Hz. Finally, band 3 is labdadiene with *m/z* (rel. int.); 302 (20), 137 (100), 123 (50), 109 (35), 95 (73), 81 (70), 69 (55), 55 (48), 41 (50). ^{1}H ($CDCl_3$): δ 0.74, 0.84 and 0.90 (*s*, each 3H, CH_3, 18, 19, 20), 1.04-2.51 (*m*, 14H, CH_2, *CH*, 1, 2, 3, 5, 6, 7, 9, 10, 11), 3.45 (*s*, 2H, CH_2, 14), 4.39 (*s*, 1H, CH_2, 17), 4.88 (*s*, 1H, CH_2, 17), 6.78 (*t*, 1H, *CH*, 12), 9.42 (*s*, 1H, *CHO*, 15) and 9.67 (*s*, 1H, *CHO*, 16) (Figure 1c).

The MTD was isolated by our laboratory. Briefly, air-dried rhizomes of alpinia (1000 g) were extracted with ethanol (1.5 L) for 2 days at room temperature. After evaporation of the solvent extract was obtained this dissolved in distilled water (300 mL) and defatted with hexane (300 mL). The defatted aqueous extract was further fractionated with chloroform ($CHCl_3$) (200 mL) and then EtOAc (200 mL). The EtOAc fraction was subjected to glass chromatography column containing silica gel (Silica gel 60N, particle size 63-120 µm, 70-230 mesh ASTM) and eluted with petroleum ether: $CHCl_3$ (0-100%) to afford three fractions. 2,5-bis (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5- dihydrofuran, HREIMS *m/z* 285.1 [M^+] (calcd for $C_{18}H_{22}O_3$, 286.16). IR *v* (KBr) cm^{-1}: 669, 1,646, 2,341, 2,359. ^{1}H-NMR (500 MHz, MeOD-d_4): 3.83 (*q*, 3H, OCH_3, J = 5.5 Hz, 9), 5.62 (*d*, 1H, *OCH*, 2), 6.24 (*d*, 1H, *CH*, 7), 6.86 (*d*, 1H, *CH*, 6), 7.58 (*s*, 1H, *CH*, 5), 7.59 (*d*, 1H, *CH*, 4), 7.14 (*q*, 1H, *CH*, J = 5.0 Hz, 1), 7.41 (*t*, 1H, *CH*, J = 7.0 Hz, 3), 7.36 (*t*, 1H, *CH*, J = 4.0 Hz, 8). ^{13}C-NMR (500 MHz, MeOD-d_4): 57.01 (C-9), 89.42 (C-2), 102.78 (C-7), 120.05 (C-6), 128.63 (C-5), 129.14 (C-4), 129.99 (C-3), 130.55 (C-1), 136.66 (C-8) (Figure 1d).

The TMOQ was also isolated by our laboratory. The seeds (100 g) of alpinia were extracted with 500 mL of methanol by maceration at room temperature for 2 days. After filtration, the filtrate was evaporated to dryness to give 21.6 g of dark syrupy extract. The extract was suspended in distilled water (500 mL) and partitioned with hexane (500 mL) and EtOAc (500 mL). The EtOAc extract (11.07 g) was subjected to glass chromatography column containing silica gel, eluted with methanol (MeOH) in dichloromethane (CH_2Cl_2) in a step gradient manner from 1% to 50% to obtain four fractions. Fraction 4 was further purified by the same column and condition as described above. (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine, HREIMS *m/z* 317.55 [M^+] (calcd for $C_{22}H_{39}N$, 317.2). IR *v* (KBr) cm^{-1}: 1,024, 1,121, 1,456, 1,507, 1,541, 1,558, 1,646, 1,698, 1,748, 2,360, 2,927, 3,448. ^{1}H-NMR (500 MHz, MeOD-d_4): 0.75 (*q*, 3H, CH_3, J = 10.5 Hz, 20), 0.85 (*t*, 3H, CH_3, J = 1.5 Hz, 18), 0.89 (*q*, 3H, CH_3, J = 4.0 Hz, 21), 0.94 (*t*, 3H, CH_3, J = 2.5 Hz, 1), 1.09 (*t*, 2H, CH_2, J = 2.5, 8), 1.17 (*q*, 2H, CH_2, 11), 1.22 (*q*, 2H, CH_2, J = 1.5 Hz, 14), 1.28 (*s*, 2H, CH_2, 14), 1.39 (*q*, 1H, *NCH*, J = 2.5 Hz, 12), 1.43 (*q*, 2H, CH_2, J = 2.2 Hz, 4), 1.58 (*t*, 3H, CH_3, J = 2.0 Hz, 19), 1.64 (*q*, 2H, CH_2, J = 4.5 Hz, 7), 1.75 (*q*, 2H, CH_2, J = 2.0 Hz, 5), 2.00 (*d*, 2H, NCH_2, 13), 2.26 (*d*, 2H, CH_2, 6), 2.47 (*t*, 2H, NCH_2, J = 5.5 Hz, 17), 3.27 (*t*, 2H, CH_2, J = 8.0 Hz, 22), 6.02 (*q*, 1H, *CH*, 3) and 6.36 (*d*, 1H, *CH*, 2). ^{13}C-NMR (500 MHz, MeOD-d_4): 14.00 (C- 21), 15.48 (C-20), 20.16 (C-4), 20.39 (C-19), 22.37 (C-18), 24.53 (C-5), 31.77 (C-7), 34.02 (C-15), 34.50 (C- 1), 37.79 (C-6), 39.90 (C-16), 40.36 (C-8), 42.03 (C-11), 43.39 (C-14), 55.96 (C-9), 63.34 (C-12), 65.83 (C-17), 68.81 (C-13), 108.85 (C-22), 122.20 (C-2), 135.00 (C- 3), 150.77 (C-10) (Figure 1e).

2.4. *Inhibition by alpinia compounds of melanogenesis*

2.4.1. *Cell culture*

Murine B16F10 melanoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 U/100 µg/mL) at 37°C in a humidified atmosphere containing 5% CO_2.

2.4.2. *Cell viability*

Cell viability was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, as described by Campos *et al*. (*23*). Briefly, B16F10 cells were plated at a density of 7 × 10^3 cells/well in a 96-well plate. After 48 h of culture, cells were exposed to varying concentrations of alpinia compounds (100 and 200 µg/mL) or 500 µM kojic acid and incubated for an additional 48 h at 37°C. Following incubation, the medium was removed, and the cells were washed twice with phosphate buffer and incubated with MTT solution (0.5 mg/mL) for 3 h at 37°C. The medium was discarded, and 200 µL of ethanol was added. The absorbance of each well was measured at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4.3. *Measurement of melanin content*

Melanin content was determined as described by Yoon *et al*. (*24*). In brief, B16F10 cells were plated at a density of 7 × 10^3 cells/well in a 96-well plate. After 48 h of culture, cells were exposed to varying concentrations of alpinia compounds (20 and 50 µg/mL) or 500 µM kojic acid. After 1 h, 100 µM isobutyl-1-methylxanthine (IBMX) was added and incubated for an additional 48 h at 37°C. The cells were washed twice with phosphate buffer, and then dissolved in 100 µL NaOH (1 N) containing 10% dimethyl sulfoxide (DMSO). Samples were incubated at 80°C for 1 h, and mixed to solubilize the melanin. The optical density of the mixed homogenate was measured at 490 nm. To measure the

amount of melanin in each experiment, the total amount of melanin (100%) produced during the experimental period was considered as the control group, and the rate of inhibition in the treatment groups was calculated in proportion to this standard.

2.4.4. *Intracellular tyrosinase activity*

Tyrosinase activity was determined as described by Li *et al*. (*25*) with slight modifications. B16F10 cells were plated at a density of 7×10^3 cells/well in a 96-well plate. After 48 h of culture, cells were exposed to varying concentrations of alpinia compounds (20 and 50 μg/mL) or 500 μM kojic acid. After 1 h, 100 μM IBMX was added and incubated for an additional 48 h at 37°C. The cells were then washed with ice cold phosphate buffer and lysed with phosphate buffer (pH 6.8) containing 1% Triton-X (90 μL/well). The plates were frozen at -80°C for 30 min. After thawing and mixing, 10 μL of 1% L-DOPA was added to each well. Following incubation at 37°C for 2 h, the absorbance was measured at 490 nm.

2.5. *Inhibition by alpinia compounds of ROS and NO production in cultured 3T3-L1 adipocytes*

2.5.1. *Cell culture and differentiation*

3T3-L1 cells were grown to confluency in Dulbecco's modified eagle's medium (DMEM) with 2% glutamine and 10% calf serum (CS) (v/v). Two days after reaching confluency, the cells were stimulated to differentiate into adipocytes by growing for an additional two days in DMEM that contained 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, and 10 μg/mL insulin. Cells were then maintained in DMEM with 10% FBS and 10 μg/mL insulin for another two days, followed by culturing in DMEM with only 10% FBS for an additional four days. At that time, greater than 90% of the cells were differentiated 3T3-L1 adipocytes with accumulated lipid droplets. Differentiated 3T3-L1 cells were treated with different concentrations of the test compounds and maintained at 37°C in a humidified incubator containing an atmosphere of 5% CO_2 throughout the experiments.

2.5.2. *3T3-L1 cell viability assay*

Cell viability was measured using the MTT assay. The 3T3-L1 preadipocytes were seeded at a density of 1×10^4 cells per well in 96-well plates and incubated in culture medium. The cells were then treated with various concentrations of the tested compounds range from 100 and 250 μg/mL. After 72 h, the cells were incubated in the dark with an MTT solution for 4 h at 37°C. The supernatants were aspirated, DMSO was added to each well, and the plates were agitated to dissolve the formazan crystal product. Absorbance was then measured at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc, Hercules, CA, USA). The percentage of viable cells was calculated by defining the cell viability without treatment as 100%.

2.5.3. *Measurement of intracellular ROS*

The 3T3-L1 cells were plated at a density of 2×10^6 cells/mL onto 96-well plates and cultured to confluency and differentiation as the method described above. ROS production was detected by nitro blue tetrazolium (NBT) assay (*26*). NBT is reduced by ROS to a dark-blue, insoluble form of NBT called formazan. After differentiation, cells were incubated the tested compounds at the various concentrations range from 10 and 20 μg/mL for 24 h. Cells then were incubated for 90 min with 100 μL in phosphate-buffered saline (PBS) containing 0.2% NBT. The dark-blue formazan was dissolved in 50% acetic acid, and its absorbance was determined at 570 nm.

2.5.4. *Measurement of NO production*

Cells were seeded and differentiated on 96-well plates as described previously. The nitrite formation (NO_2) assay used has been reported earlier by Fang *et al*. (*27*). After differentiation, cells were incubated with alpinia compounds, 10 and 20 μg/mL, for 24 h. Individual supernatants (100 μL) and Griess reagent (100 μL, 1:1 mixture (v/v) of 1% sulfanilamide and 0.1% napthyethylenediamine dihydrochloride in 5% H_3PO_4) were mixed in a separate 96 well plates and incubated for 10 min at room temperature. Absorbance was measured at 540 nm using a microplate spectrophotometer and nitrite concentrations were estimated using a standard curve generated for $NaNO_2$.

2.6. *Statistical analysis*

Data are expressed as mean values with their standard errors. Statistical comparisons were performed by oneway ANOVA followed by Duncan's multiple-range test. Statistical analysis was conducted using SAS (release 9.2; SAS Institute, Cary, NC, USA) and $p < 0.05$ was considered significant.

3. Results

3.1. *Anti-melanogenic effects of alpinia compounds*

Many cosmetic and pharmaceutical products have tried to find safe and effective inhibitors for melanogenesis. Especially, inhibitors for melanin formation and tyrosinase can be used for the inhibition of cellular pigmentation since melanin producing process involved a series of enzymatic and non-enzymatic oxidation (*11*). Thus, to examine the effect of alpinia compounds on

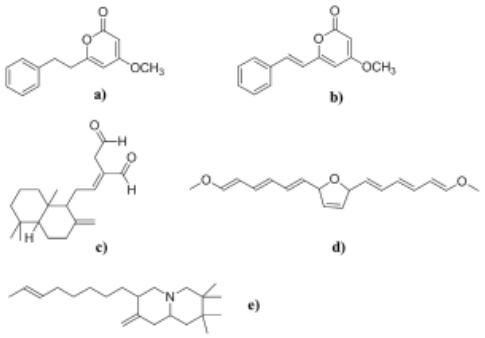

Figure 1. Chemical structure of DDK (1a), DK (1b), Labdadiene (1c), MTD (1d), TMOQ (1e).

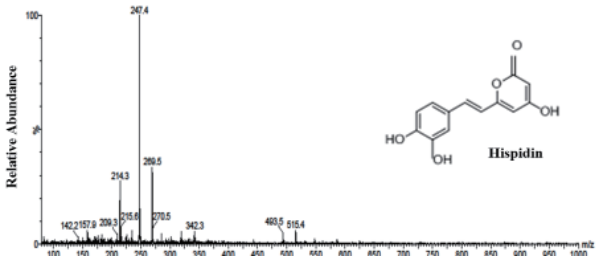

Figure 2. MS/MS spectra of hispidin (the peak 247.4) obtained by LC/MS analysis of the rat liver microsomes incubated with DK in the presence of NADPH-generating system.

melanogenesis, we measured both melanin content and intracellular tyrosinase activity.

First of all, we examined their effect on the viability *per se* of B16F10 melanoma cells by incubating them with each test compound for 48 h. As shown in Figure 3, none of these compounds (up to 100 μg/mL) significantly affected the cell viability.

3-Isobutyl-1-methylxanthine (IBMX) is an often used potent melanogenic stimulator (*28*), which up-regulates tyrosinase(s). Thus, in the presence of IBMX, we evaluated the anti-melanogenic activity of alpinia components.

3.1.1. *Melanin content*

The B16F10 melanoma cells were treated with each alpinia compound (20-50 μg/mL) in the presence of IBMX for 48 h. As shown in Figure 4, all treatments significantly ($p < 0.05$) reduced the melanin content. At 50 μg/mL MTD, DK, DDK, labdadiene, hispdin and TMOQ inhibited the melanin synthesis by 79%, 72%, 68%, 67%, 63% and 59%, respectively. Under same conditions, kojic acid (500 μM), a positive control, inhibited the melanin synthesis by 50.5%.

3.1.2. *Inhibition of intracellular tyrosinase activity*

To evaluate whether alpinia compounds inhibit intracellular tyrosinase activity or not, the B16F10 melanoma cells were treated the tested compounds (20-50 μg/mL) for 48 h, followed by incubation with L-DOPA. After treatment at 20 μg/mL, tyrosinase

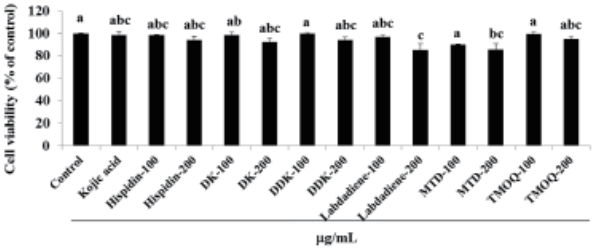

Figure 3. Effect of alpinia compounds on B16F10 melanoma cell viability. B16F10 cells were treated with tested compounds at either 100 or 200 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Reported values are the means ± SE ($n = 3$). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

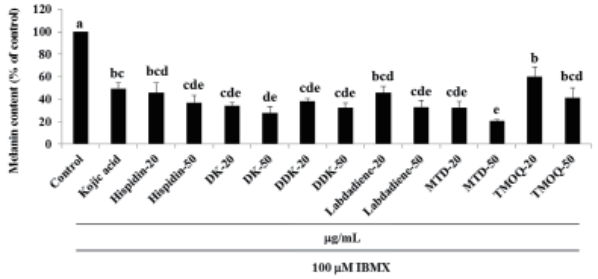

Figure 4. Effect of alpinia compounds on melanin production in B16F10 melanoma cells. B16F10 cells were treated with tested compounds at either 20 or 50 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. Reported values are the means ± SE ($n = 3$). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

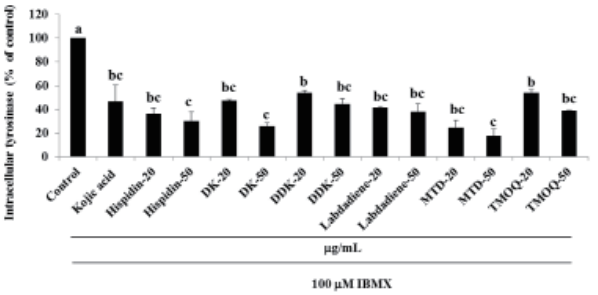

Figure 5. Effect of alpinia compounds on intracellular tyrosinase activity in B16F10 melanoma cells. B16F10 cells were treated with tested compounds at either 20 or 50 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. Reported values are the means ± SE ($n = 3$). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

activity was inhibited by 76%, 64%, 59%, 52%, 46%, and 46% with MTD, hispidin, labdadiene, DK, TMOQ and DDK, respectively. At 50 μg/mL, all the tested compounds inhibited tyrosinase more potently than the positive control kojic acid (53%) (Figure 5). Among these compounds, MTD, DK and hispidin inhibited more strongly the tyrosinase activity by 83%, 74% and 70%, respectively, than labdadiene, TMOQ and DDK which inhibited the tyrosinase by 62%, 61% and 55%, respectively, without damaging the melanocyte viability.

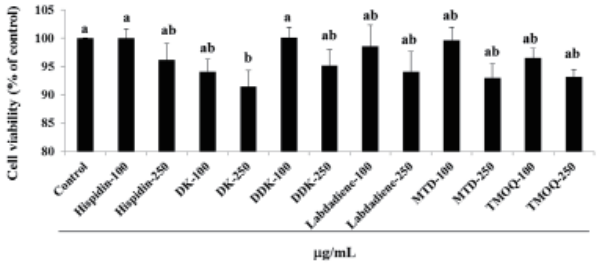

Figure 6. Effect of alpinia compounds on 3T3-L1 viability. 3T3-L1 cells were treated with tested compounds at either 100 or 250 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Reported values are the means ± SE (n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

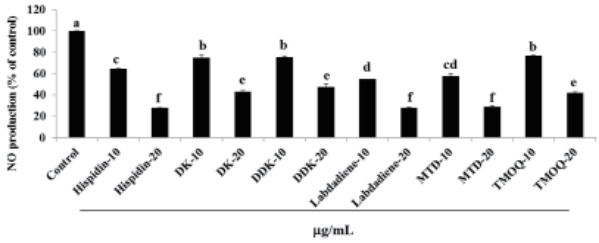

Figure 8. Effects of alpinia compounds on intracellular NO. 3T3-L1 adipocytes were treated with tested compounds at either 10 or 20 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Reported values are the means ± SE (n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

3.2. *Effects of alpinia on ROS and NO production in cultured 3T3-L1 adipocytes*

3.2.1. *Effect of alpinia compounds on 3T3-L1 cell viability*

To examine whether alpinia compounds have effect on cells, 3T3-L1 cells were incubated with them (100-250 μg/mL) for 72 h. At 100 μg/mL none of compounds showed any effect on the cell viability. At 250 μg/mL, DK, MTD, TMOQ, labdadiene, DDK and hispidin only slightly decreased cell viability by 9, 7, 7, 6, 5 and 4%, respectively (Figure 6).

3.2.2. *Measurement of intracellular ROS*

We found that alpinia compounds strongly inhibited the ROS production in cultured adipocytes. At 20 μg/mL ladadiene, MTD, TMOQ and hispidin in particular strongly inhibited the ROS production by 64%, 60%, 53% and 46%, respectively (Figure 7). DK and DDK showed to prevent ROS production with percentage inhibition values of 44% and 42%.

3.2.3. *Measurement of intracellular NO*

The NO production was also strongly inhibited by

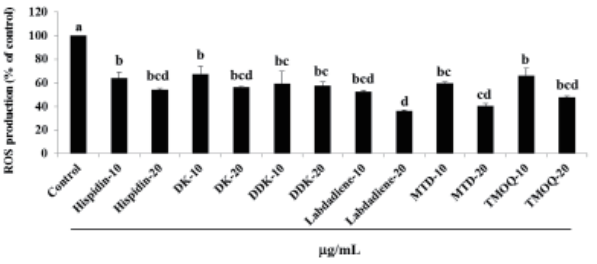

Figure 7. Effects of alpinia compounds on intracellular ROS production. 3T3-L1 adipocytes were treated with tested compounds at either 10 or 20 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Reported values are the means ± SE (n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

alpinia compounds (Figure 8). For example, labdadiene, hispidin and MTD at 20 μg/mL reduced the NO production by 72%, 72%, and 71%, respectively, while TMOQ, DDK and DK were less effective (by 58%, 57%, and 52%, respectively).

4. Discussion

We revealed here that several alpinia compounds are potent anti-melanogenic agents. At the concentration of 50 μg/mL, MTD, DK, hispidin and labdadiene in particular are effective anti-melanogenic agents, reducing both melanin content and intracellular tyrosinase by more than 50%. Furthermore, they are the stronger anti-melanogenic agents than kojic acid, which is a standard skin-lightening agent for treating melanogenesis and related diseases.

Furthermore, this study revealed that these alpinia compounds strongly inhibit both ROS and NO production in the cultured 3T3-L1 adipocytes, suggesting that alpinia is a good source for anti-oxidants, blocking both ROS and NO production.

In conclusion, the present study demonstrates that alpinia rhizome or seed is a safe and effective source for treating ROS and melanogenesis, without causing any cytotoxicity in either adipocytes or melanocytes. Among the compounds being tested, MTD, labdadiene, hispidin and DK are among four potent inhibitors of ROS and NO production in 3T3-L1 adipocytes. They also reduce the melanogenesis, suggesting that a common mechanism is involved in both anti-ROS/NO production and anti-melanogenesis. Nevertheless it is clear that alpinia extracts could be utilized for treating obesity as well as a skin-whitening cosmetic agent for the treatment of hyper-pigmentation.

Regarding the detailed molecular mechanism underlying anti-melanogenesis by these alpinia compounds, several lines of indirect evidence suggest that these herbal compounds could block melanogenesis and NO production through their PAK1-blocking activity: *i*) a variety of herbal compounds such as CAPE, curcumin, and FTY720 that block the

oncogenic/ageing kinase PAK1 also strongly inhibit the melanogenesis by down-regulating the oncogenic and melanogenic transcription factor MITF which is essential for the expression of tyrosinase gene(s) (*15*); *ii*) among these alpinia compounds, at least hispidin, DK and DDK are known to inhibit directly PAK1 *in vitro* (*3*); *iii*) NO production requires PAK1 (*19*); *iv*) we have recently confirmed biochemically by shRNA-induced silencing of PAK1 gene in melanocytes that PAK1 is essential for the melanogenesis in skin cells (Be Tu *et al*., manuscript submitted). However, it is also possible that some of these alpinia compounds might block PAK4 as well, because contribution of PAK1 to the melanogenesis is estimated only 50%, and some of these compounds, in particular MTD, inhibits the melanogenesis clearly by far more than 50%. Thus, it would be worth testing the anti-PAK1/anti-PAK4 activity of MTD, labdadiene and TMOQ from alpinia seeds in cell culture. In addition, so far nobody has studied the possible role of PAK4 in either obesity or ROS/NO production in adipocytes. Perhaps it could be worth testing this possibility as well.

Finally, it might be worth noting that years ago several compounds such as galangin and kaempheride from *Alpinia officinarum* were found to inhibit the melanogenesis of melanoma cell line (*29*). However, the anti-melanogenic compounds from *Alpinia zerumbet* studied here have nothing to do with those (from *Alpinia officinarum*) previously reported. These findings indicate a surprising diversity of their ingredients among the alpinia family, and yet they share the common cosmetic/pharmacological property.

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