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ORIGINAL ARTICLE

Anti-Inflammatory and Anti-Melanogenic Effects of Major Leaf Components of *Alpinia zerumbet* var. *excelsa*

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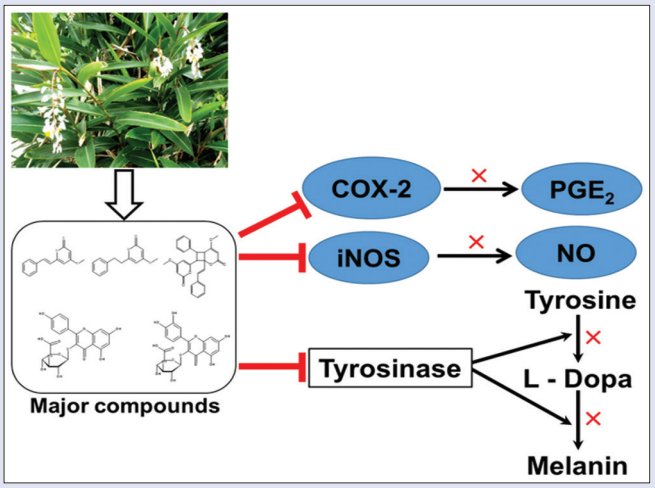
ABSTRACT

Background: The leaves of *Alpinia zerumbet* var. *excelsa* (*alpinia*) are used to prepare traditional food items and folk medicines. **Objective:** This study was designed with a view to explore the anti-inflammatory and anti-melanogenic potentials of major components of *alpinia* leaves. **Materials and Methods:** Anti-inflammatory effects of leaf-derived compounds were primarily assessed with protein denaturation and proteinase assay. Their inhibitory effects on nitrite accumulation and prostaglandin E2 (PGE_2) production in lipopolysaccharide-induced RAW 264.7 cells were also evaluated. For anti-melanogenic assays, all the compounds were tested on α-melanocyte-stimulating hormone-stimulated B16F10 cells and on mushroom tyrosinase *in vitro*. Their cytotoxicity was evaluated using fibroblast cell line 3T3 L-1 and brine shrimps. **Results:** Five compounds, 5,6-dehydrokawain, dihydro-5,6-dehydrokawain, (*E*)-5-methoxy-8-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-7-phenyl-1-styryl-2-oxabicyclo[4.2.0]oct-4-en-3-one (AS-2), kaempferol 3-O-*β*-D-glucuronide (KOG), and quercetin 3-*O*-*β*-D-glucuronide (QOG) were purified from the leaves. Of which, AS-2 and QOG were purified for the first time. All compounds significantly inhibited albumin denaturation and proteinase activity. AS-2, KOG, and QOG remarkably inhibited nitric oxide formation with IC_{50} values of 8.2, 13.3, and 12.6 μM, respectively, in RAW 264.7 cells. They also inhibited PGE_2 production with IC_{50} values 19.8-23.7 μM. They showed anti-melanogenic effects reducing tyrosinase activity (IC_{50} values 29.6-112.5 μM) and melanin formation (IC_{50} values 30.8–164.4 μM) in B16F10 cells, and inhibiting mushroom tyrosinase (IC_{50} values 61.5-456.4 μg/ml). **Conclusion:** Taken together, major components of *alpinia* leaf could be utilized as a potent herbal drug and food supplement with therapeutic prospects against inflammatory disorders and hyperpigmentation.

Key words: *Alpinia zerumbet*, inflammation, melanogenesis, protein denaturation, RAC/CDC42-activated kinase 1

SUMMARY

- In the present study, we have purified five major compounds from *alpinia* leaves and described their anti-inflammatory and melanogenesis inhibitory effects *in vitro*. Two compounds were purified for the first time from this plant. They all were found to have significant inhibitory effects on inflammation and melanogenesis. These effects might be attributed to their RAC/CDC42-activated kinase 1 blocking properties. Moreover, these compounds are not cytotoxic to the normal cells and to the small animal model brine shrimps. Hence, *alpinia* leaves could be a good choice for developing non-toxic herbal drug to treat inflammatory disorders and skin-hyperpigmentation.



Abbreviations used: HPLC: High performance liquid chromatography; DK: 5,6-dehydrokawain; DDK: Dihydro-5,6-dehydrokawain; AS-2: (*E*)-5-methoxy-8-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-7-phenyl-1-styryl-2-oxabicyclo[4.2.0]oct-4-en-3-one; KOG: Kaempferol 3-*O*-*β*-D-glucuronide; QOG: Quercetin 3-*O*-*β*-D-glucuronide; PGE_2: Prostaglandin E2; PAK1: RAC/CDC42-activated kinase 1; UV: Ultra violet; α-MSH: α-Melanocyte-stimulating hormone; COX-2: Cyclooxygenase-2; iNOS: Inducible Nitric Oxide Synthase; PDGF: Platelet Derived Growth Factor; MITF: Microphthalmia-Associated Transcription Factor; TRP: Tyrosinase Related Protein; ATCC: American Type Culture Collection; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal bovine serum; CS: Newborn calf serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DMSO: Dimethyl sulfoxide; NO: Nitric oxide; LPS: Lipopolysaccharide; DMRT: Duncan's multiple range test; SPSS: Statistical Package for the Social Sciences.

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INTRODUCTION

Inflammation is nowadays implicated as one of the important pathophysiological mechanisms underlying several diseases or disorders including diabetes, cardiovascular diseases, cancer, arthritis, neurodegenerative diseases, and autoimmune diseases. Chronic inflammation is thought to be one of the major triggering factors for several pigmentary disorders.[1,2] In post-inflammatory sites on the

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skin, hyperpigmentation occurs mainly due to high accumulation of histamine and arachidonic acid metabolites.[3] Prostaglandin E2 (PGE_2) is also found to be increased in inflamed skin regions.[3] These all are termed as inflammatory mediators which accelerate melanin production in melanocytes and transfer the melanin pigments to the neighboring keratinocytes.[3] Moreover, ultraviolet (UV) radiation, a major factor for skin hyperpigmentation, also causes inflammation through releasing inflammatory mediators[1,4] and thus, it causes photoaging, hyperpigmentation, wrinkles, and loss of skin firmness and flexibility.[5] Recently, an oncogenic/aging kinase RAC/CDC42-activated kinase 1 (PAK1) has been reported to be a key factor for inflammation and melanogenic signaling pathways.[6,7] As a downstream effector, PAK1 activates cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) and regulates the pathways involved in pain and inflammation.[6,7] In the melanogenic pathway, it is activated by the major growth hormone platelet-derived growth factor, which in turn activates beta-catenin and microphthalmia-associated transcription factor.[8] They induce the expression of tyrosinase and tyrosinase-related protein genes essential for melanin formation.[8] Therefore, PAK1 blocking components might be effective for inhibiting inflammation and melanogenesis, and they could be a good therapeutic choice for treating inflammation, skin hyperpigmentation, and other inflammatory disorders as well.

Alpinia zerumbet is a perennial zingiberous plant, which is widely distributed in subtropical and tropical regions worldwide. Two varieties of this plant, *tairin* (*A. zerumbet* (Pers.) B. L. Burtt and R. M. Sm. var. *excelsa* Funak and T. Y. Ito) and *shima* (*A. zerumbet* (Pers.) B. L. Burtt and R. M. Sm.), are found in Okinawa, Japan, and they differ from each other with their morphological features and biological activities.[9] *Tairin* variety is found to be grown there in abundant. However, leaves from both varieties are conventionally used there to prepare rice cakes, herbal teas, and to flavor the noodles. Japanese people also prepare leaf decoctions from *A. zerumbet* for treating fever and the common cold.[10,11] This plant was investigated for its pharmacological benefits against obesity, diabetic, hypertension, and atherosclerosis,[12-15] but no studies have examined yet on its anti-inflammatory effects. Moreover, the phytochemicals isolated from this plant were reported to have PAK1 blocking activities.[16-18] Hence, we predicted that it might have inhibitory effects on inflammatory disorders, and this study was undertaken to investigate the anti-inflammatory and anti-melanogenic effects of major leaf components of *A. zerumbet* var. *excelsa* (*alpinia*).

MATERIALS AND METHODS

Cell culture and reagents

Murine macrophage cell line (RAW 264.7) and melanoma cell line (B16F10) (American Type Culture Collection [ATCC], Manassas, Virginia, USA) were cultured in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, Saint Louis, Missouri, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Victoria, Australia) and 1% penicillin/streptomycin (10,000 U/ml and 100 µg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. The mouse embryonic fibroblast cell line 3T3-L1 (ATCC) was cultured in the same conditions using 10% newborn calf serum (CS, HyClone, Victoria, Australia) instead of FBS. DMEM without phenol red was purchased from Thermo Fisher Scientific (Massachusetts, USA). Casein, Griess reagents, 70% perchloric acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Chicken egg albumin and trypsin were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). All other chemicals and reagents used in this study were of analytical grade and obtained from either Wako Pure Chemical Industries Ltd. (Osaka, Japan) or Kanto Chemical Co. Inc., (Tokyo, Japan).

Plant material

Alpinia leaves were collected from the field of Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan. The plant material was identified by Prof. Shinkichi Tawata, and the voucher specimens (PRC-2016-01) are stored at PAK Research Center, University of the Ryukyus. The materials were collected in a plastic bag and used in the extraction process soon after the collection.

Extraction and purification of dehydrokawain, dihydro-5,6-dehydrokawain, and (*E*)-5-methoxy-8-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-7-phenyl-1-styryl-2-oxabicyclo[4.2.0]oct-4-en-3-one

Fresh leaves were cut into small pieces and washed with tap water. The leaves were then kept at room temperature for 24 h and placed into an oven at 45°C for 48 h. The dried leaves were then used to make a powder using a kitchen blender. Approximately 30 g of leaf powder was soaked in 400 ml acetone for 48 h, and then the extracts were collected through filtration. Extraction was performed twice, and following that, the solvent was evaporated with reduced pressure at 4°C temperature using a rotary evaporator. Dried extracts were dissolved in water (200 ml), then partitioned with ethyl acetate, and the final 940.1 mg (3.13%) of ethyl acetate extract was collected. The ethyl acetate extract was subjected to high-performance liquid chromatography (HPLC) using a semi-preparative C18 column (Inertrsil ODS-3, 250 mm × 10 mm, GL Sciences Inc., Tokyo, Japan), and three compounds: 5,6-dehydrokawain (DK), dihydro-5,6-dehydrokawain (DDK), and (*E*)-5-methoxy-8-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-7-phenyl-1-styryl-2-oxabicyclo[4.2.0]oct-4-en-3-one (AS-2) [Figure 1a-c] were purified. Water (solvent A) and methanol (solvent B) were used as mobile phases with a flow rate of 2.5 ml/min. HPLC conditions were as follows: 0–44 min, 60% solvent B; 0-30 min, 75% solvent B; 30.01–37 min, 100% solvent B; and 37.01–44 min, 60% solvent B.

Extraction and purification of kaempferol 3-*O*-*β*-D-glucuronide and quercetin 3-*O*-*β*-D-glucuronide

Fresh leaves (250 g) were directly placed into 500 ml boiling water, extracted for 15 min, and then the extract was cooled at room temperature. The extract was then filtered and partitioned with *n*-hexane, dichloromethane, and chloroform. Afterward, the extract was fractioned continuously with ethyl acetate and butanol to produce a crude extract (ALEB). The ALEB extract was chromatographed with Sephadex LH-20 (GE Healthcare Life Sciences) with ethanol/acetone gradient elution to yield two fractions. Fraction 1 was subjected to preparative thin-layer chromatography with butanol/acetic acid/water solvent (6:1:2). Kaempferol 3-*O*-*β*-D-glucuronide (KOG) and quercetin 3-*O*-*β*-D-glucuronide (QOG) [Figure 1d and e] were purified through HPLC using a mobile phase of 0.1% acetic acid in distilled water (solvent A) and 0.1% acetic acid in methanol (solvent B). The gradient elution was performed as follows: 0–27 min, 10% solvent B; 27–30 min, 90% solvent B.

Spectral details and structure determination of the compounds

The chemical structures of the purified compounds were identified using nuclear magnetic resonance (NMR) and mass spectrometry data. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker

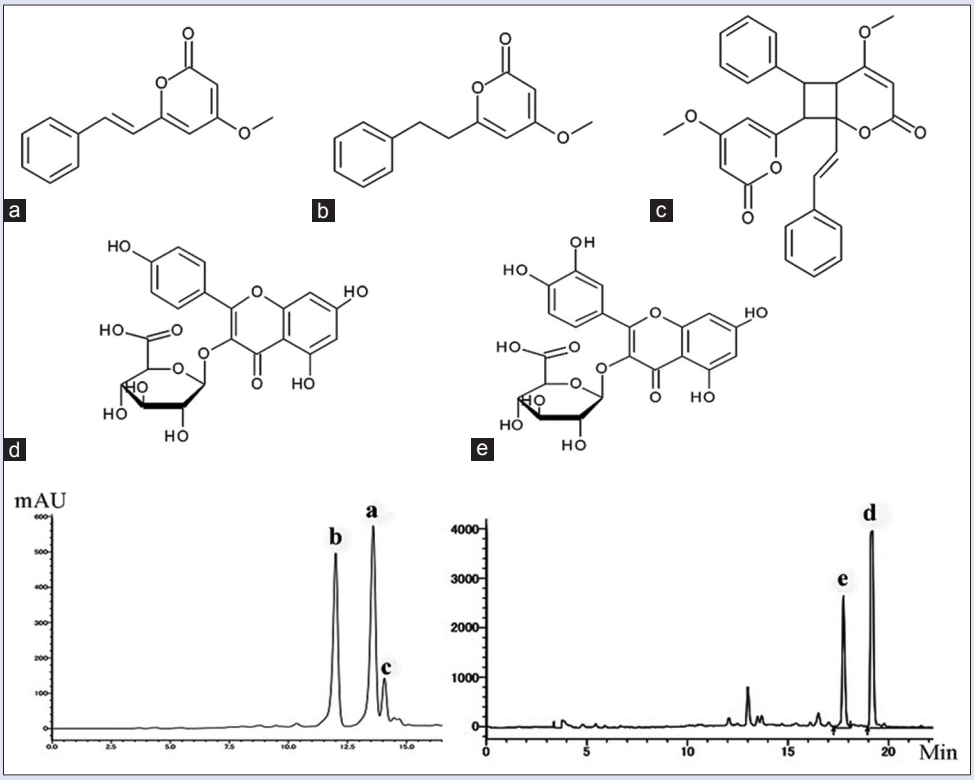

Figure 1: Chemical structures of 5,6-dehydrokawain (DK) (a), dihydro-5,6-dehydrokawain (DDK) (b), (*e*)-5-methoxy-8-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-7-phenyl-1-styryl-2-oxabicyclo[4.2.0]oct-4-en-3-one (AS-2) (c), kaempferol 3-O-β-D-glucuronide (KOG) (d), and quercetin 3-O-β-D-glucuronide (QOG) (e), and their corresponding peaks in HPLC (high performance liquid chromatography) chromatograms

Biospin GmbH (Rheinstetten, Germany) in $CDCl_3$. Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane. DK and DDK [Figure 1a and b] were confirmed by comparing their physical data (mass spectra, ^1H and ^{13}C NMR spectra) with the values reported in the literature.[19] Similarly, KOG [Figure 1d] was identified by comparing its mass spectra as well as ^1H and ^{13}C NMR spectra to the published data.[16] Spectral details for AS-2 [Figure 1c] are as follows: electrospray ionization mass spectrometry (ESI-MS, *m/z)* = 457.1 $[M + H]^+$ (calculated for $C_{28}H_{25}O_6$, 457.49); ^1H NMR (500 MHz, $CDCl_3$): δ 7.45-7.27 (10H, aromatic), 6.98 (1H, d, J = 15.9 Hz), 6.62 (1H, d, J = 15.9 Hz), 5.94 (1H, d, J = 2.2 Hz), 5.37 (1H, d, J = 2.2 Hz), 5.32 (1H, s), 4.39 (1H, dd, J = 11.0, 9.8 Hz), 4.19 (1H, d, J = 11.0 Hz), 3.75 (3H, s), 3.62 (1H, d, J = 9.8 Hz), 3.30 (3H, s). ^{13}C NMR (500 MHz, CDCl3): δ 170.5 (C-4), 169.9 (C-4′), 164.56 (C-2), 163.85 (C-2′), 158.64 (C-6), 135.89 (C-9′), 135.62 (C-9), 131.52 (C-8′), 128.75, 128.47, 128.3, 127.84 (C-11,12,13,11′,12′,13′), 127.54 (C-10/14), 126.89 (C-10′/14′), 124.41 (C-7′), 102.67 (C-5), 91.8 (C-3′), 88.73 (C-3), 79.43 (C-6′), 55.88 (C-15), 55.4 (C-15′), 54.5 (C-7), 45.73 (C-5′), 39.22 (C-8). Spectral details for QOG [Figure 1e] are as follows: ESI-MS (*m*/z) = 479.0 $[M + H]^+$ (calculated for $C_{21}H_{19}O_{13}$, 479.36); ^{1}H-NMR (400 MHz, MeOD-d_4): δ 3.27-3.51 (H2″, H3″, H4″, H5″, m, 4H), 5.32 (H1″, d, J = 7.2, 1H), 6.22 (H8, d, J = 2.0 Hz, 1H), 6.42 (H6, d, J = 2.0 Hz, 1H), 6.82 (H5′, d, J = 8.4 Hz, 1H), 7.41 (H6′, dd, J = 8.4, 2.0 Hz, 1H), 7.43 (H2′, d, J = 2.0 Hz, 1H); ^{13}C NMR (400 MHz, MeOD-*d*4): δ 177.6 (C-4), 170.2 (C-6′), 164.6 (C-7), 161.6 (C-5), 157.1 (C-2), 156.7 (C-9), 149.1 (C-4′), 145.4 (C-3′), 133.7 (C-3), 121.3 (C-1′), 122.1 (C-6′), 116.4 (C-2′), 115.6 (C-5′), 104.3 (C-10), 101.5 (C-1″), 99.1 (C-6), 94.0 (C-8), 76.5 (C-3″), 76.2 (C-5″), 74.2 (C-2″), 71.8 (C-4″). Spectral data of AS-2 and QOG were found to be in agreement with the values reported in the literature.[20,21]

Cell growth/viability assay

Cell viability for B16F10, RAW 264.7, and 3T3-L1 cells were evaluated through MTT assay.[22] Briefly, all the cells were seeded in 48-well plates for 24 h. B16F10 cells were seeded with a density of 1×10^4 cells/well, whereas RAW 264.7 and 3T3-L1 cells were seeded with a density of 1×10^5 cells/well. The culture medium was then replaced with fresh medium containing various sample concentrations. Indomethacin was used as control in Raw 264.7 cells, whereas kojic acid was used as control in B16F10 cells. Both indomethacin and kojic acid were used as negative control in 3T3-L1 cell culture. After adding the samples with desired concentrations, RAW 264.7 cells were incubated for 24 h, while B16F10 and 3T3-L1 cells were incubated for 48 h. The supernatant was then removed and aliquots of 100 µl MTT solution (0.5 mg/ml in phosphate buffer saline [PBS]) were added to each well, and the plate was incubated again at 37°C for 3 h in humidified conditions supplemented with 5% CO_2. After 3 h, 500 µl of DMSO was added to each well, and the plate was shaken for 30 min to dissolve the formazan crystals. Finally, the absorbance was measured at 570 nm wavelength and cell viability was calculated from the absorbance of treated versus untreated cells.

Protein denaturation inhibition assay

Protein denaturation inhibition assay was carried out using egg albumin according to the previously described method[23] with slight modifications. Briefly, the reaction mixture contains 0.1 ml egg albumin, 0.7 ml PBS and 0.5 ml of varying concentrations of different compounds. Negative control contains similar amount of distilled water instead of the compounds. The mixtures were then incubated at 37°C for 15 min and heated at 70°C for 5 min. After cooling, the absorbance was measured at 660 nm using microplate reader. Diclofenac sodium was used as positive

control. The inhibition percentage of protein denaturation was calculated according to the following formula:

Percentage of inhibition = ([1−[A/B]] ×100)

Where, A = absorbance of the test sample, and B = absorbance of the negative control.

Proteinase inhibitory activity

It was done according to the method described by Govindappa *et al.*[24] The reaction mixture (2 ml) was consisting of 0.08 mg trypsin, 1 ml of 20 mM Tris hydrochloric acid buffer (pH 7.4) and 1 ml of test compounds at desired concentrations. The mixture was then incubated at 37°C for 5 min. After that, 0.8% (w/v) casein (1 ml) was added and the mixture was incubated for additional 20 min. A volume of 1 ml of 70% perchloric acid was added to terminate the reaction. Finally, the reaction mixture was centrifuged, and the absorbance of supernatant was measured at 210 nm. The experiment was performed twice in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated by using the following formula.

Percentage of inhibition = ([Absorbance of control − Absorbance of sample]/Absorbance of control) ×100

Nitrite accumulation in RAW 264.7 cells

RAW 264.7 cells were seeded in 24-well plates with a density of 2×10^5 cells/well for 24 h at 37°C in humidified conditions supplemented with 5% CO_2. After aspirating the medium, 400 µl DMEM (without phenol red) containing 10% FBS and 1% penicillin/streptomycin was added to each well. Cells were incubated for 24 h with lipopolysaccharide (LPS) (500 ng/ml final concentration) and various concentrations of the test compounds. Indomethacin at 100 µM concentration was used as a positive control. Nitrite accumulation in each well was determined using the Griess reagent. The culture supernatant (100 µl) was mixed with equal volumes of Griess reagent and incubated for 10 min at room temperature. The absorbance was recorded at a wavelength of 540 nm and compared to the standard curve prepared using a series of known concentrations of sodium nitrite.

Measurement of prostaglandin E2 in RAW 264.7 cells

PGE_2 is one of the major inflammatory mediators produced following COX-2 activation. RAW 264.7 cells (2×10^5 cells/well) were seeded in 24-well plates for 24 h. Cells were then treated with LPS (500 ng/ml) and different concentrations of the test compounds for 24 h. Indomethacin at 10 µM concentration was used here as a positive control. The PGE_2 levels were determined in the culture supernatant using a PGE_2 enzyme-linked immunosorbent assay kit (Cayman Chemical Co., Ann Arbor, Michigan, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm with a microplate reader.

Determination of melanin content in B16F10 cells

In vitro melanin content was measured according to a method described previously.[9] In brief, the B16F10 melanoma cells were seeded with a density of 2×10^4 cells/well in 24-well plates and incubated overnight. The cells were then exposed to various sample concentrations for 48 h in the presence of 100 nM α-melanocyte stimulating hormone (α-MSH). Then, the cells were washed twice with PBS and lyzed with 400 µl of 1 N NaOH containing 10% DMSO and incubated for 1 h at 80°C. The optical density of the mixed homogenate was measured at a wavelength of 405 nm using a microplate reader. The rates of melanin inhibition in the treatment groups were calculated in comparison to the control group.

Intracellular tyrosinase inhibition in B16F10 cells

Intracellular tyrosinase activity in B16F10 cells was measured with a modified way of a previous method.[9] Cells were seeded at a density of 2×10^4 cells/well in 24-well plates for 24 h and exposed to different sample concentrations for 48 h in the presence of 100 nM α-MSH. The cells were then washed with ice-cold PBS and lyzed with 500 µl of phosphate buffer containing 1% TritonX and then frozen at −80°C for 30 min. After thawing and mixing, 100 µl of 0.5% L-DOPA was added to each well. Following incubation at 37°C for 2 h, the absorbance was measured at 492 nm wavelength. Intracellular tyrosinase activity was calculated in comparison to the α-MSH treated control group.

Mushroom tyrosinase assay

The effects of different compounds on cell free mushroom tyrosinase activity were determined spectrophotometrically with a modified method.[9] Tyrosinase activity was measured using tyrosine as a substrate. In brief, 120 µl of phosphate buffer (20 mM, pH 6.8), 20 µl of sample and 20 µl of mushroom tyrosinase (500 units/ml in 20 mM phosphate buffer) were added to each well of a 96-well plate. The reaction mixture was pre-incubated at room temperature for 10 min, and then reaction was initiated by adding 20 µl of 0.85 mM L-tyrosine solution to each well, and incubation was continued for next 20 min at room temperature. Kojic acid was used as a standard tyrosinase inhibitor. The amount of dopachrome formed due to the action of tyrosinase enzyme was determined at 470 nm in a microplate reader. The percentage of tyrosinase inhibition was calculated as follows:

Tyrosinase inhibition (%) = ([A − B]−[C − D])/(A − B) ×100, where A is the absorbance of the control with the enzyme, B is the absorbance of the control without the enzyme, C is the absorbance of the test sample with the enzyme, and D is the absorbance of the test sample without the enzyme.

Brine shrimp toxicity assay

Toxicity of different compounds were evaluated with brine shrimp (*Artemia salina*) toxicity assay.[25] Briefly, 200 mg of dried brine shrimp eggs (Tetra Brine Shrimp Eggs, Spectrum Brands Holdings, Inc., Kanagawa, Japan) was hatched in artificial seawater supplemented with dried yeast with suitable aeration at 28°C in room conditions for 48 h. Then, active nauplii were transferred to a glass petri dish-containing artificial seawater. Samples were primarily dissolved in DMSO and then diluted with artificial seawater. Samples were added to each well (12–15 larvae per well in 100 µl artificial seawater), and then the well plate was kept at 20°C. The final concentration of DMSO was 1% (v/v), and DMSO at 1% was used in the control wells. After 48 h of incubation, the number of dead larvae in each well was counted using magnifying lenses (5× or 10×). Methanol (50 µl) was then added to each well, and the total number of larvae in each well was counted after 1 h. The percentage of viability for each well was then calculated.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Data were analyzed by paired Student's *t* test with $P < 0.05$ and $P < 0.01$ indicating significance. However, Duncan's multiple range test was carried out using IBM SPSS Statistics 24 (IBM Corporation, Armonk, NY, USA) at 5% probability level.

RESULTS AND DISCUSSION

Inhibitory effects on protein denaturation and proteinase activity

External stimuli such as strong acid or base, inorganic salt, organic solvent or thermal treatment denature the proteins structurally, and in

turn proteins are failed to explore their biological potency.[26,27] Denatured proteins are one of the major causes of inflammation, rheumatic disorders, cataract, and Alzheimer's disease.[26,28] Moreover, proteinases secreted from the neutrophils and leukocytes at inflammatory site may cause tissue damage and further inflammation, and proteinase inhibitors are thought to be effective for significant level of tissue protection.[24,29] Herein, we studied *alpinia* leaf derived compounds against protein denaturation and proteinase activity *in vitro*. We found that all the compounds exhibited potential inhibitory effects on both protein denaturation and proteinase activity in dose-dependent manner [Figure 2a and b]. DDK showed significant inhibition on egg albumin denaturation with IC_{50} value of 14.5 μM, whereas the IC_{50} values for DK, AS-2, KOG, and QOG were 14.9, 25.5, 25.4, and 20.4 μM. On the other hand, DK showed stronger proteinase inhibitory activity with IC_{50} value 39.9 μM followed by DDK, AS-2, QOG, and KOG (IC_{50} values are 43.8, 44.5, 56.6, and 63.2 μM, respectively). All the compounds were found to be more effective in both protein inhibition assay and proteinase assay compared to the positive control, diclofenac sodium (IC_{50} values are 94.6 and 113.3 μg/ml, respectively). These findings, therefore, reveal the prospect of *alpinia* leaf components to be further studied against inflammation using another model and developed into effective pharmaceutical agents as well.

Anti-inflammatory effects in lipopolysaccharide-stimulated RAW 264.7 cells

Inflammatory mediators such as nitric oxide (NO), cytokines, chemokines and eicosanoids are produced during inflammation for generating a series of protective responses. NO is considered to be a major pro-inflammatory agent[30] which is involved in other diseases including asthma, rheumatoid arthritis, and atherosclerosis.[31] Similarly, PGE_2 is another well-studied pro-inflammatory mediator which contributes to the development of the cardinal signs of acute inflammation through redness, swelling, and pain.[32] Nowadays, NO and PGE_2 inhibition have been revealed as an effective treatment option for inflammation and related disorders.[33,34] In this study, we tested the effects of purified compounds on formation of NO and PGE_2 in RAW 264.7 cells following LPS-induced inflammation. Firstly, all the compounds were evaluated for their effects on RAW 264.7 cell viability through MTT assay. It was revealed that the compounds did not show cytotoxicity on RAW 264.7 cells, whereas they showed a degree of cell proliferation at specific concentrations [Figure 3a], suggesting that pro-proliferative activity in RAW 264.7 cells. Therefore, the nontoxic concentrations of the five compounds were used in the subsequent experiments to determine their effects on NO and PGE_2 formation. We measured the amount of NO in culture supernatants of LPS-induced RAW 264.7 cells in the presence or absence of each compound. All the compounds inhibited NO production in a concentration-dependent manner [Figure 4a]. DK and DDK showed moderate inhibitory effects (IC_{50} values-27.6 and 28.1 μM, respectively), whereas KOG and QOG showed stronger inhibition (IC_{50} values-13.2 and 12.5 μM, respectively) on NO formation. However, AS-2 showed superior performance to all other compounds with IC_{50} value 8.2 μM against NO production. Next, we measured PGE_2 after treating the cells with different compounds at the indicated concentrations. PGE_2 production was found to be gradually decreased with the increasing concentrations of the compounds compared to the LPS-stimulated control cells [Figure 4b]. AS-2, KOG, and QOG showed significant effects, whereas DK and DDK were found to be less potent with regard to PGE_2 inhibition. The IC_{50} concentrations for PGE_2 inhibition by DK, DDK, AS-2, KOG, and QOG were 69.6, 92.4, 19.8, 22.7, and 23.7 μM, respectively. AS-2 showed the highest anti-inflammatory effects compared to all other tested compounds, inhibiting both NO and PGE_2 significantly. In LPS-treated macrophages, NO and PGE_2 are produced from L-arginine and arachidonic acid, respectively, and these reactions are catalyzed by transcriptional activation of iNOS and COX-2 gene, respectively.[35,36] Therefore, inhibition of NO and PGE_2 production by *alpinia* leaf compounds might be attributed to their downregulatory effects on iNOS and COX-2 activity. This notion warrants further research to explore the molecular mechanisms associated with the anti-inflammatory activities of *alpinia* leaf-derived compounds.

Anti-melanogenic effects

Overproduction and subsequent accumulation of melanin (hyperpigmentation) cause several aesthetic problems such as freckles, age spots, melanoderma, and senile lentigo. Melanin biosynthesis (melanogenesis) is mostly regulated by the rate limiting enzyme tyrosinase. During melanogenesis, reactive oxidants including hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) are produced, causing oxidative stress in melanocytes.[37] This oxidative stress subsequently leads to chronic inflammation,[38] which in turn could mediate skin hyperpigmentation and cancer. As *alpinia* leaf compounds exhibited anti-inflammatory actions, we hypothesized that they would also exhibit anti-melanogenic potential through inhibition of tyrosinase activity and decreasing melanin content in B16F10 cells. They were found to be non-toxic to B16F10 cells [Table 1] and therefore, used at 10, 20, 50, and 100 μM concentrations to assess their anti-melanogenic effects. As shown in Table 1, all the compounds reduced melanin content dose-dependently. Of the five compounds tested, AS-2, KOG, and QOG were found to be more potent in inhibiting cellular melanin content with IC_{50} values of 30.8, 72.6, and 63.1 μM, respectively. In contrast, the apparent IC_{50} values for DK and DDK were calculated 164.4 and 139.4 μM, respectively.

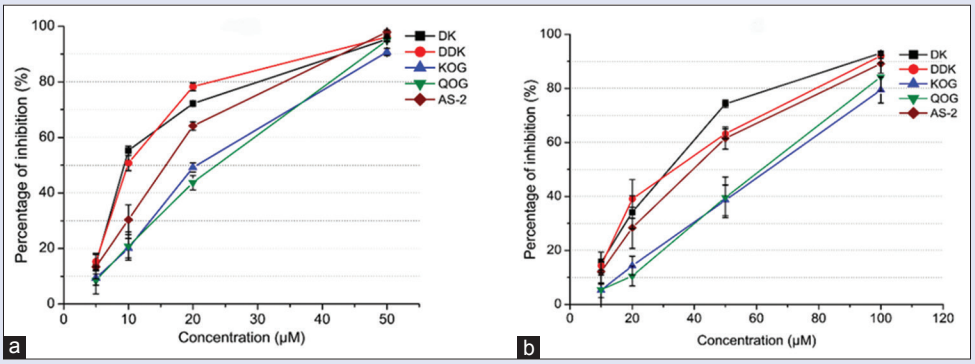

Figure 2: Protein denaturation inhibition effects (a) and proteinase inhibitory activities (b) of the compounds. Each data point represents mean ± standard error ($n = 6$)

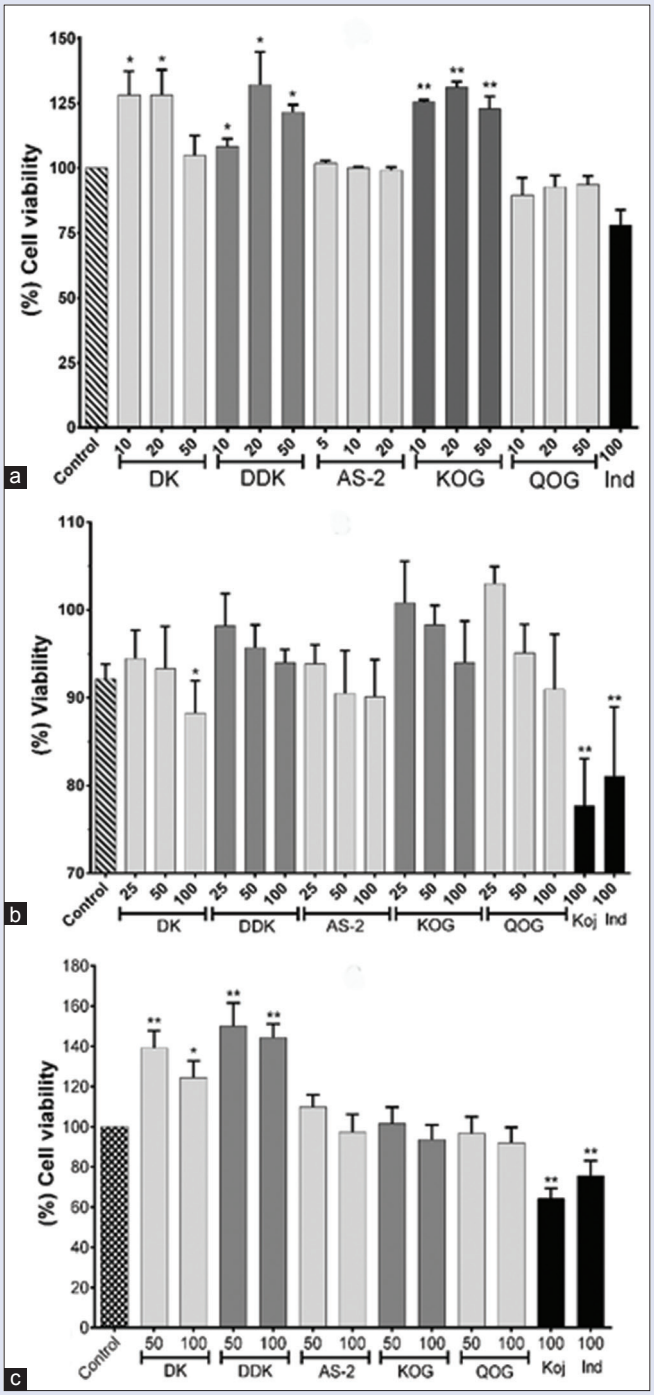

Figure 3: Cytotoxic effects of *alpinia* leaf compounds on RAW 264.7 cells (a), brine shrimps (b), and on 3T3-L1 fibroblast cells (c). The results are mean ± standard error, and the asterisks denote significant differences compared to the control group (*$P < 0.05$; **$P < 0.01$)

Furthermore, all the compounds downregulated intracellular tyrosinase activity as shown in Table 1. AS-2, KOG, and QOG demonstrated superior inhibitory action on the tyrosinase enzyme with IC_{50} values of 29.6, 63.6, and 54.0 μM, respectively. DK and DDK also inhibited cellular tyrosinase with IC_{50} values of 106.9 and 112.5 μM, respectively. In this study, AS-2, KOG, and QOG showed the highest anti-melanogenic effects without interfering with the viability of B16F10 cells. They also inhibited mushroom tyrosinase activity *in vitro* [Figure 5]. Of these, DK showed stronger effects on mushroom tyrosinase with IC_{50} value of 61.5 μg/ml, whereas the IC_{50} values for DDK, KOG, QOG, and AS-2 are 243.9, 422.2, 456.4, and 123.1 μg/ml, respectively. All the compounds showed weak inhibitory effects on mushroom tyrosinase compared to their inhibition on cellular tyrosinase. For the screening of potential skin-whitening agents, tyrosinase activity evaluation is thought to be important, and the cellular tyrosinase assay is a more reliable assay than the cell-free mushroom tyrosinase assay in this regard.[39] However, mushroom tyrosinase is found in the cytosol while tyrosinase in melanocytes is membrane bound, that's why anti-tyrosinase effects of *alpinia* compounds on these tyrosinases may not be the same.[40]

Toxic effects of the compounds

Since toxicity is the major concern to use natural compounds for medicinal/food purposes, we here tested *alpinia* leaf components on 3T3-L1 fibroblast cells and on brine shrimps. The brine shrimp is a small animal that is widely used as a model to determine the toxicity of heavy metals, pesticides and plant extracts/compounds.[41] The effects of different compounds on brine shrimps and on 3T3-L1 cells after 48 h of incubation are shown in Figure 3b and c. DK at ≥100 μM concentration showed toxic effects on brine shrimps, whereas all other compounds at tested concentrations were found to be nontoxic. They also did not show cytotoxic effects on 3T3-L1 cells compared to the control. However, DK and DDK showed pro-proliferative effects on 3T3-L1 cells increasing the cell proliferation significantly. On the other hand, kojic acid and indomethacin at 100 μM concentration showed significant cytotoxicity on both 3T3-L1 cells and on brine shrimps. These results indicate that *alpinia* compounds could be used as a safe natural therapeutic source.

From this study, we found that *alpinia* leaf components inhibit tyrosinase enzyme and eventually reduce melanin content in B16F10 cell culture. They also inhibit tyrosinase enzyme activity *in vitro*. In addition, these compounds inhibit PGE_2 production and NO formation in RAW 264.7 cells, which might be due to their down-regulating effects on COX-2 and iNOS gene expression, respectively. Previously, our group concentrated on direct PAK1 inhibitory effects of *alpinia* compounds, and confirmed that DK, DDK, labdadiene (12-labdadiene-15,16-dial), MTD ((1E,3E,5E)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran), TMOQ ((E)-2,2,3,3-Tetramethyl-8-methylene-7-(oct-6-en-1-yl) octahydro-1H-quinolizine), and KOG directly inhibit PAK1 *in vitro* with the IC_{50} values of 17, 10.3, 52.1, 58.6, 49.3, and 39.3 μM, respectively.[16-18] Out of all compounds, DK and DDK showed the highest PAK1 blocking activities, which are due to the presence of α-pyrone ring and methoxy group in their structure.[18] However, AS-2 contains more α-pyrone ring and methoxy group in their structure than that of DK and DDK. On the other hand, chemical structure of QOG and KOG are quite similar. We, therefore, can hypothesize that AS-2 and QOG may also inhibit PAK1 directly, although we are currently testing their *in vitro* PAK1 blocking effects. Taking the present study and the previous report of PAK1 blocking effects of *alpinia* compounds into account, we here propose the possible pathways on their anti-melanogenic and anti-inflammatory actions based on Song *et al.*[6], Nguyen *et al.*[7], and Maruta *et al.*[8] [Figure 6]. Their anti-melanogenic effects might be induced in two different ways-direct inhibition of tyrosinase enzyme activity or down-regulation of tyrosinase enzyme through blocking its downstream effector PAK1. Similarly, their inhibition on PGE_2 and NO formation could be due to their PAK1 blocking effects and eventual down-regulation of COX-2 and iNOS activity. These proposed actions of *alpinia* leaf compounds could be further confirmed using molecular biology study in combination with molecular modeling techniques.

Table 1: Effects of different compounds on cell proliferation, melanin content, tyrosinase activity in B16F10 cells

Compounds (μM)	Melanin content (percentage of control)	Tyrosinase activity (percentage of control)	Cell proliferation (percentage of control)
KOG			
10	82.6±1.5^{a,b}	79.3±0.9^{b}	106.2±0.9*
20	79.0±1.3^{c}	65.0±1.5^{c}	104.5±1.6
50	47.8±0.8^{k}	45.8±1.1^{g,h}	104.2±0.8
100	42.4±1.8^{l}	39.9±1.0^{i}	99.3±1.2
QOG			
10	79.9±0.8^{b,c}	81.7±2.3^{a,b}	104.0±2.1
20	70.1±1.1^{f,g}	67.4±2.9^{c}	102.3±1.7
50	43.1±1.1^{l}	42.5±1.3^{h,i}	100.6±0.8
100	39.1±0.6^{m}	28.7±1.1^{j}	101.1±1.4
DK			
10	74.0±0.9^{d,e}	85.5±2.0^{a}	104.0±2.0
20	76.9±1.1^{c,d}	78.5±2.2^{b}	100.5±2.0
50	71.7±1.2^{e,f}	64.3±2.7^{c,d}	94.2±2.8*
100	60.1±0.8^{h}	54.5±1.0^{e,f}	92.8±1.7*
DDK			
10	74.8±1.3^{d,e}	86.9±2.5^{a}	106.1±1.0*
20	74.0±0.8^{d,e}	79.1±2.7^{b}	102.5±2.1
50	72.6±1.2^{e,f}	66.8±1.8^{c}	96.5±2.0
100	56.3±1.0^{i}	55.9±1.0^{e,f}	91.2±1.5*
AS-2			
10	68.4±0.3^{g}	59.2±0.7^{d,e}	105.0±0.7
20	50.9±0.6^{j}	53.6±0.3^{e,f}	103.1±0.9
50	33.8±0.6^{n}	40.7±0.4^{h,i}	104.2±1.3
100	17.3±0.7^{o}	20.0±0.8^{k}	100.1±2.7
Kojic acid			
500	84.0±0.7^{a}	51.0±4.1^{f,g}	98.5±1.3

Values are expressed as mean ± standard error (n=6). The different letters within column 3 and 4 show significant differences among the different group means by Duncan's multiple range test at 5% probability. Asterisks indicate a significant difference compared to the control group (*P < 0.05). KOG: Kaempferol 3-O-β-D-glucuronide; QOG: Quercetin 3-O-β-D-glucuronide; DK: 5,6-Dehydrokawain; DDK: Dihydro-5,6-dehydrokawain; AS-2: (*E*)-5-methoxy-8-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-7-phenyl-1-styryl-2-oxabicyclo[4.2.0]oct-4-en-3-one

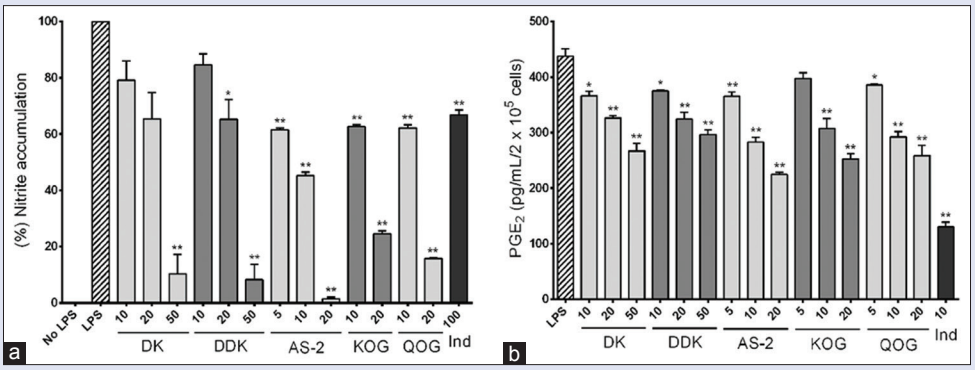

Figure 4: Inhibitory effects of the compounds on nitrite accumulation (a) and PGE2 production (b) in lipopolysaccharide (LPS)-induced RAW 264.7 cells. After seeding, the cells were incubated with LPS (500 ng/ml) and varying concentrations (μM) of the tested compounds for 24 h. Data are shown as mean ± standard error of two experiments. Asterisks indicate significant differences compared to the LPS-treated control group (*P < 0.05; **P < 0.01)

CONCLUSION

We have found that the major compounds of *alpinia* leaves have considerable inhibitory effects on both inflammatory and melanogenic pathways. Importantly, this dual inhibitory action is reported here for the first time. All the compounds exhibited anti-inflammatory activities through inhibiting protein denaturation, proteinase activity, and the formation of inflammatory mediators-NO and PGE_2. They also inhibited cellular tyrosinase activity and melanin formation in cultured B16F10 cells, and mushroom tyrosinase activity *in vitro* demonstrating their anti-melanogenic potentials. These compounds can inhibit PAK1, a downstream effector of inflammatory and melanogenic signaling cascades, and hence, their anti-inflammatory and anti-melanogenic effects are thought to be attributed to their PAK1 blocking activities. In addition, all the compounds were found to be non-toxic to the brine shrimps, 3T3-L1 cells, B16F10 cells, and RAW 264.7 cells. In conclusion, *alpinia* leaf components can function as a source for potent and safe natural therapies to treat inflammation and hyperpigmentation, and the leaves could be commercially utilized to develop herbal supplements as well as value-added foodstuffs.

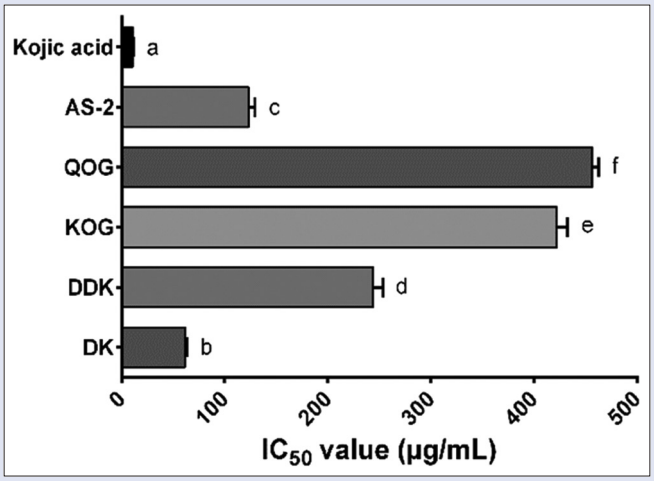

Figure 5: Effects of the compounds on mushroom tyrosinase activity *in vitro*. Each bar represents mean ± standard error of three experiments. The different letters indicate significant differences among compounds by Duncan's multiple range test at 5% probability level

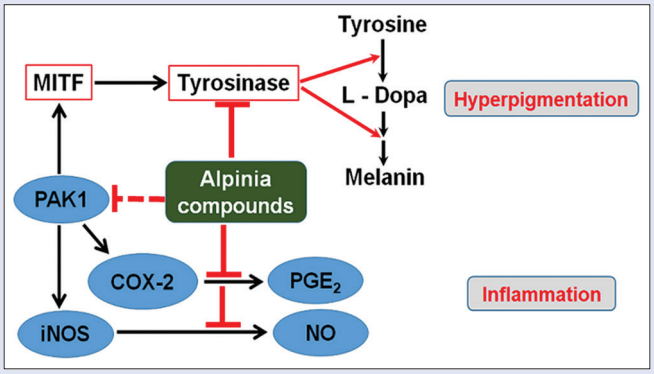

Figure 6: Role of PAK1 in melanogenic and inflammatory signaling pathways, and the probable inhibitory actions of *alpinia* components on inflammation and melanogenesis

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Nil.

Conflicts of interest

There are no conflicts of interest.

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