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Bioassay-Guided Isolation and Identification of Compounds from Arecae Pericarpium with Anti-inflammatory, Anti-oxidative, and Melanogenesis Inhibition Activities

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Abstract – This study describes the anti-inflammatory, anti-oxidant, and melanogenesis inhibition activities of methanol extract and various organic solvent fractions of Arecae Pericarpium. We examined the inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cells, 1,1-diphenyl-2-picrylhydrazine (DPPH) scavenging activity, mushroom tyrosinase inhibition activity and melanin contents. The study showed that, among all tested fractions, methylene chloride fraction showed the strongest inhibition of LPS-induced NO production in RAW 264.7 cells (IC_{50} value 8.89 μg/mL) and DPPH radical scavenging activity (EC_{50} value 21.39 μg/mL). Methylene chloride and ethyl acetate fractions similarly inhibited mushroom tyrosinase activity. Methanol extract exhibited strongest reduction of melanin content in B16F10 melanoma cells. Based on the bioactivity assay results, methylene chloride and ethyl acetate fractions were further separated. Eight phenolic compounds were isolated, which are dimeric syringol (**1**), catechol (**2**), 4-hydroxybenzaldehyde (**3**), vanillin (**4**), 4-hydroxyacetophenone (**5**), apocynin (**6**), protocatechuic acid (**7**) and 4-hydroxybenzoic acid (**8**). Among the isolated compounds tested, catechol showed the strongest inhibition of LPS-induced NO production in RAW 264.7 cells. Catechol also showed the concentration-dependent NF-κB inhibition activity. Arecae Pericarpium might have potentials to be developed as anti-inflammatory agent or dermatological product for skin-whitening agent.
Keywords – Arecae Pericarpium, Anti-inflammatory, Antioxidative, Melanogensis Inhibition, Bioassay-guided isolation

Introduction

Arecae Pericarpium is the fruit husk of *Areca catechu* L. (Arecaceae). According to the *Compendium of Materia Medica,* this herb has been used in Traditional Chinese Medicine (TCM) for abdominal distension, constipation, and edema treatment. Its combination with other herbs in Huo Xiang Zheng Qi (藿香正气片) formula is used to treat summer heat-dampness diseases and gastrointestinal cold, to cure abdominal distension, vomiting, and diarrhea.[1] Modern investigations showed that Arecae Pericarpium has a fungicidal activity against *Colletotrichum gloeosporioides* Penz. *in vitro* and in mango fruit medium.[2] Arecae Pericarpium also showed the dose-dependent antioxidative activity in human hepatocarcinoma $HepG_2$ cell line. The methanol extract of Arecae Pericarpium showed the stronger antioxidant activity compared to other parts of the *Areca catechu* (L.) plant.[3] The limited number of studies on Arecae Pericarpium suggests that Arecae Pericarpium has high potential to be further studied for its bioactivity. Thus, in this present report, we investigated the anti-inflammatory, free radical scavenging, and melanogenesis inihibition activity of Arecae Pericarpium.

The potential anti-inflammatory activity of Arecae Pericarpium was screened by examining the ability of its fractions in suppressing LPS-induced nitric oxide (NO) production in RAW 264.7 murine macrophage cells. Macrophage plays a central role in a host's defense system against bacterial infection through phagocytosis, cytotoxicity, and intracellular killing.[4,5] Stimulation of murine macrophages by LPS results in the expression of iNOS and increased NO production which plays a critical role in macrophage activation and is associated with acute and chronic inflammations.[5] The elevated NO production is examined with Griess reaction by quantifying the nitrite level in the conditioned medium of RAW 264.7 cells treated with LPS. This cell-based assay has been used for drug screening and the evaluation of potential inhibitors

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of the pathways leading to the induction of iNOS and NO production.[6]

Melanin is secreted by melanocytes and determines the color of skin and hair in mammalians. It protects the skin by absorbing UV sunlight and removing reactive oxygen species (ROS).[7] The excessive level of melanin pigmentation causes various dermatological disorders including hyperpigmentations such as senile lentigo, melasma, postinflammatory melanoderma, freckles, ephelide, age spots and sites of actinic damage which can give rise to esthetic problems.[8] Many studies have been conducted to find depigmenting agents from natural resources that can be used for the long-term without side effects. Arecae Semen has been reported to have anti-inflammatory and melanogenesis inhibitory activity.[9] Thus, in this study Arecae Pericarpium was also tested to investigate whether it also has potential to inhibit melanogenesis.

Experimental

General experimental procedures – All organic solvents used for extraction, column chromatography and high-performance countercurrent chromatography (HPCCC) were of analytical grades. HPLC-grade acetonitrile and methanol were used for HPLC analysis and preparative HPLC. Distilled water was used for all solutions and dilutions. Silica gel 60 (0.063 - 0.200 mm) was used for open column silica chromatography separation. Silica gel 60 F254 20 × 20 cm was used for thin-layer chromatography. Diaion®-HP20 (polystyrene adsorption resin) was used for open column chromatography separation.

HPLC analyses were carried out on Hitachi L-6200 and Agilent 1100 instruments. Low resolution electrospray ionization source (ESI) LC/MS data were recorded on Agilent Technologies 1200 HPLC coupled with Agilent Technologies 6130 Quadrapole mass spectrometer. Columns used for analysis are iNNO C18 column, LUNA C18 column and Zorbax SB-C18 column. Spectrum high-performance countercurrent chromatography (HPCCC) from Dynamic Extractions Ltd with tubing id 1.6 mm, total volume 135.5 mL, and sample loop 6 mL was used for CCC separation. Preparative HPLC separation was performed using a Hitachi JP/L-7100 equipped with Hitachi L-4000 UV detector. The column used for semi-preparative separation was RSil C18 column (250 mm × 10 mm id, 10 μm particle size). The NMR analyses were recorded on Bruker Avance 500 and 600 spectrometers. ^{1}H and ^{13}C NMR spectra were measured in a DMSO-d_6 solution at 500 and 125 MHz or 600 and 150 MHz, respectively.

Plant materials – Arecae Pericarpium cultivated in China was purchased at a local herb market in Seoul (October, 2013). Arecae Pericarpium was identified by Professor Young Bae Suh, College of Pharmacy, Seoul National University. A voucher specimen (AP201510a) was deposited in Natural Products Research Institute, College of Pharmacy, Seoul National University.

Extraction – The dry Arecae Pericarpium (5 kg) was macerated in 28 L of methanol for 3 days. The process was repeated for 3 times. The macerate was dried with a rotary evaporator under 50 ºC. The extract was then suspended with 1 L of 10% methanol and partitioned with hexane, methylene chloride (MC), ethyl acetate (EA), and *n*-butanol (BuOH) in 1:1 ratio for 3 times and each fraction was dried with a rotary evaporator. The final state of all dried fractions was viscous state.

NO inhibition and cell viability assay – The ability of tested samples in inhibiting NO production was determined by Griess reagent assay. RAW 264.7 cells were plated at density of 1×10^5 cells/well in a 24-well plate with 500 μL of culture media and incubated for 24 h. Then, cells were treated with samples in various concentration for 2 h and stimulated with LPS (1 μg/ mL) for another 18 h. An aliquot of cell-free medium (100 μl/ well) was then removed to a 96-well plate and Griess reagent (100 μl/well) was added. To quantify nitrite concentration, standard nitrite solutions were prepared and the absorbance of the mixture was determined at 540 nm with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA, USA). Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric assay. AMT, an iNOS inhibitor, was used as a positive control (10 μM). Cells treated with vehicle alone were used as a control.

NF-κB secretory alkaline phosphatase (SEAP) reporter gene assay – Reporter enzyme activity was measured by cell-based assay system for NF-κB activity monitoring.[10] The pNF-κB-SEAP-NPT plasmid that permits expression of the SEAP reporter gene in response to the NF-κB activity and contains the neomycin phosphotransferase (NPT) gene for geneticin resistance in host cells was constructed and transfected into murine macrophages. Transfected RAW 264.7 cells were plated at density of 1×10^5 cells/well in a 24-well plate with 500 μL of geneticin-added culture medium and incubated at 37 ºC for 24 h. Cells were treated with samples in various concentrations for 2 h and stimulated with LPS (1 μg/mL) for 18 h. Aliquots of cell-free medium (120 μL) of each treatment was transferred to 1.5 mL vial and heated at 65 ºC for 6 min and given an assay buffer (2 M dietha-

nolamine, 1 mM $MgCl_2$, 500 μM 4-methylumbelliferyl phosphate (MUP)) in the dark at 37 ºC for 1 h. The fluorescence from the product of the SEAP/MUP was measured using a 96-well microplate fluorometer at an excitation of 360 nm and emission of 449 nm. TPCK (20 μM) was used as a positive control.

DPPH radical scavenging assay – DPPH (1,1-diphenyl-2-picrylhydrazine) radical scavenging activity was determined with methods as described by Prieto *et al*.[11] Ascorbic acid was used as a positive control. Samples dissolved in DMSO were diluted with MeOH in 96-well plate with total volume 100 μl/well. DPPH 0.2 mM solution (100 μl) was added to each well and incubated in dark room for 30 minutes. Diluted compounds without DPPH were used as blank samples. The absorbance was measured at 540 nm with a microplatereader.

Mushroom tyrosinase assay – Various concentrations of samples dissolved in DMSO were diluted 500-fold in 0.1 M sodium phosphate buffer (pH 6.8). One hundred microliters of diluted samples and 50 μl of 1.5 mM L-tyrosin were transferred to a 96-well plate and pre-incubated for 10 min. Then, 50 μl of mushroom tyrosinase (125 unit/mL in 0.1 M sodium phosphate buffer) was added to each well and incubated at 37 ºC for 30 min. The absorbance was determined at 490 nm with a microplate reader. Kojic acid was used as positive control.

Cell viability assay – The effect of prepared samples on B16F10 melanoma cell viability was measured with MTT-based colorimetric assay. In brief, cells were seeded at density of 3×10^3 cells/well in 96-well plate and incubated for 24 h. Cells were treated with samples in various concentrations and further incubated for 3 days. On the last day, the medium was removed, replaced with MTT solution and incubated for 2 h. Mitochondrial succinate dehydrogenase in living cells are known to convert MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in DMSO and the absorbance was measured at wavelength 595 nm using a microplate fluorometer.

Melanin content assay – B16F10 melanoma cells were seeded at density of 2×10^4 cells/well in a 24-well plate and incubated for 24 h. The cells were treated with samples in various concentrations and 1 mM theophylline for 3 days. To determine the extracellular melanin content, 200 μl of cell-free medium was transferred to 96-well plate and the absorbance was determined at 490 nm with a microplate reader. To determine the intracellular melanin content, the remaining medium was removed and the cells were harvested by trypsinization (0.25% trypsin/0.02% EDTA in PBS). The harvested cells were centrifuged and solubilized in 100 μl of 1 M NaOH and heated at 60 ºC for 1 h. The absorbance was determined at 405 nm with a microplate reader.

Bioassay-guided isolation of MC fraction – The MC fraction (18 g) was subjected to silica gel column chromatography with a gradient elution of chloroform – ethyl acetate (9:1, 7:3, 5:5, 3:7, 1:9 v/v) continued with ethyl acetate – methanol (9:1, 7:3, 5:5 v/v). Six fractions were collected and the MC1 fraction was separated with silica gel column chromatography. The sample (3.3 g) was eluted with hexane-ethyl acetate-*n*-butanol with ratio (3:2:0.05) and (2.75:2.25:0.05). Seven fractions were collected from this separation (MC1A – MC1D were eluted with 3:2:0.05, and MC1E - MC1G were eluted with 2.75:2.25:0.05). MC1G was identified as dimeric syringol (**1**) (yield: 9.2 mg). MC1A fraction was subjected to Diaion HP-20 column chromatography and eluted with aqueous methanol from 20% - 100% MeOH. Catechol (**2**) (yield: 25.4 mg) was obtained from 20 and 30% MeOH elution. MC1B fraction was separated with combination of high performance counter-current chromatography (HPCCC) and preparative HPLC. HPCCC was run using HEMW solvent system (2:5:1:4 v/v/v/v). The stationary phase retention was 70.11%. Sample injection was 130.3 mg in 6 mL of solvent system mixture and detected with UV detector at 280 nm for 200 minutes. Preparative HPLC was performed with isocratic elution 18% MeOH with 0.05% formic acid under 246 nm UV detection. 4-Hydroxybenzaldehyde (**3**) (yield: 1.0 mg), vanillin (**4**), 4-hydroxyacetophenone (**5**) (yield of 4 and 5 mixture: 5.0 mg), and apocynin (**6**) (yield: 0.6 mg) were obtained from MC1B separation process.

Bioassay-guided isolation of EA fraction – The ethyl acetate fraction was subjected to Diaion® HP-20 resin column chromatography and eluted with aqueous methanol in increasing concentration from 20%, 40%, 60%, and 80% methanol. The 20% MeOH fraction showed NO inhibition activity and it was further separated by HPCCC. Several solvent systems were tested to estimate the optimum *K* values and HEMW system (2:5:2:5 v/v/v/v) was selected for HPCCC operation. The operation of HPCCC separation was conducted with UV detection set at 280 nm. 200.3 mg of sample was weighed and dissolved in 6 mL of upper phase and lower phase mixture. The operation was performed for 200 minutes. Protocatechuic acid (7) (yield: 18.7 mg) and 4-hydroxybenzoic acid (8) (yield: 3.3 mg) were obtained from the HPCCC separation.

Identification of isolated compounds – The isolated compounds were identified by NMR 500 or 600 MHz in DMSO-d_6. 1D (1H and 13C) and 2D NMR (HSQC and

HMBC) was performed at SNU National Center for Inter-University Research Facilities.

Statistical analysis – All data were derived from three independent experiments and are expressed as mean ± standard deviation (SD). Statistically significant differences between the control and experimental groups were calculated by Student's *t* test. Notes are given as (*) if $P < 0.05$, (**) if $P < 0.01$, and (***) if $P < 0.001$.

Dimeric syringol (1) – slightly yellow amorphous powder. ESI-MS *m/z* 307.1 $[M+H]^+$, *m/z* 305.1 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.35 (2H, s, H-1, H-10), 6.81 (4H, s, H-3, H-5, H-8, H-12), and 3.83 (12H, s, OCH_3) ppm; ^{13}C NMR (DMSO-d_6, 500 MHz) δ 148.3 (C-2, C-6, C-9, C-11), 134.9 (C-1, C-10), 131.4 (C-4, C-7), 104.4 (C-3, C-5, C-8, C-12) and 56.3 ppm (OCH_3).

Catechol (2) – white amorphous powder. ESI-MS *m/z* 109.1 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.82 ppm (1H, br s, H-1, H-2), 6.74 (2H, m, $J = 9.5$ Hz, 3.5 Hz, H-3, H-5), 6.58 (2H, m, $J = 9.5$ Hz, 3.5 Hz, H-4, H-6); ^{13}C NMR (DMSO-d_6, 500 MHz) δ 145.3 (C-1, C-2), 119.4 (C-4, C-5) and 115.8 (C-3, C-6).

4-hydroxybenzaldehyde (3) – white amorphous powder. ESI-MS *m/z* 121.1 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 9.76 (1H, s, H-7), 7.75 (2H, d, $J = 8.5$ Hz, H-2, H-6), 6.92 ppm (2H, d, $J = 8.5$ Hz, H-3, H-5); ^{13}C 2D NMR (DMSO-d_6, 600 MHz) δ 191.1 (C-7), 164.0 (C-4), 132.3 (C-1), 128.3 (C-2, C-6), 116.0 ppm (C-3, C-5).

Vanillic aldehyde (4) – white amorphous powder. ESI-MS *m/z* 153.1 $[M+H]^+$, *m/z* 151.0 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.73 (1H, s, H-7), 7.39 (1H, d, $J = 7.6$ Hz, H-6), 7.35 (1H, s, H-2), 6.92 (1H, d, $J = 7.6$ Hz, H-5), 3.79 ppm (3H, s, OCH_3); ^{13}C 2D NMR (DMSO-d_6, 600 MHz) δ 190.9 (C-7), 154.0 (C-4), 148.5 (C-3), 128.0 (C-1), 126.3 (C-6), 115.4 (C-5), 110.3 (C-2), 55.3 ppm (OCH_3).

4-hydroxyacetophenone (5) – white amorphous powder. ESI-MS *m/z* 137.0 $[M+H]^+$, *m/z* 134.8 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.82 (2H, d, $J = 8.6$ Hz, H-2, H-6), 6.83 ppm (2H, d, $J = 8.6$ Hz, H-3, H-5); ^{13}C 2D NMR (DMSO-d_6, 600 MHz) δ 196. 3 (C-7), 162.1 (C-4), 130.7 (C-2, C-6), 128.5 (C-1), 115.0 (C-3, C-5), 26.1 ppm (CH_3).

Apocynin (6) – off-white solid. ESI-MS *m/z* 167.1 $[M+H]^+$, *m/z* 165.1 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.49 (1H, dd, $J = 8.2$ Hz, 1.9 Hz, H-6), 7.42 (1H, d, $J = 1.7$ Hz, H-2), 6.85 (1H, d, $J = 8.2$ Hz, H-5), 3.81 (3H, s, OCH_3), 2.48 ppm (3H, s, CH_3); ^{13}C 2D NMR (DMSO-d_6, 600 MHz) δ 196. 3 (C-7), 152.0 (C-4), 147.7 (C-3), 123.4 (C-6), 114.7 (C-5), 128.5 (C-1), 110.8 (C-2), 55.3 (OCH_3), 26.1 ppm (CH_3).

Protocatechuic acid (7) – white with slightly brown colored solid. ESI-MS *m/z* 155.1 $[M+H]^+$, *m/z* 153.1 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 9.66 (1H, br s, H-4), 9.34 (1H, br s, H-3), 7.32 (1H, d, $J = 2$ Hz, H-2), 7.28 (1H, dd, $J = 8$ Hz, 2 Hz, H-6), 6.78 ppm (1H, d, $J = 8$ Hz, H-5); ^{13}C 2D NMR (DMSO-d_6, 500 MHz) δ 167.4 (C-7), 150.1 (C-4), 144.9 (C-3), 122.0 (C-6), 121.7 (C-1), 116.6 (C-2), and 115.2 ppm (C-5).

4-hydroxybenzoic acid (8) – white solid. ESI-MS *m/z* 139.1 $[M+H]^+$, *m/z* 137.0 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.26 (1H, br s, H-4), 7.79 (2H, dt, $J = 10$ Hz, 5 Hz, 3Hz, H-2, H-6), 6.82 ppm (2H, dt, $J = 10$ Hz, 5 Hz, 3 Hz, H-3, H-5); ^{13}C 2D NMR (DMSO-d_6, 500 MHz) δ 167.3 (C-7), 161.7 (C-4), 131.6 (C-2, C-6), 121.4 (C-1), and 115.2 ppm (C-3, C-5).

Result and Discussion

The ability of Arecae Pericarpium in different solvent

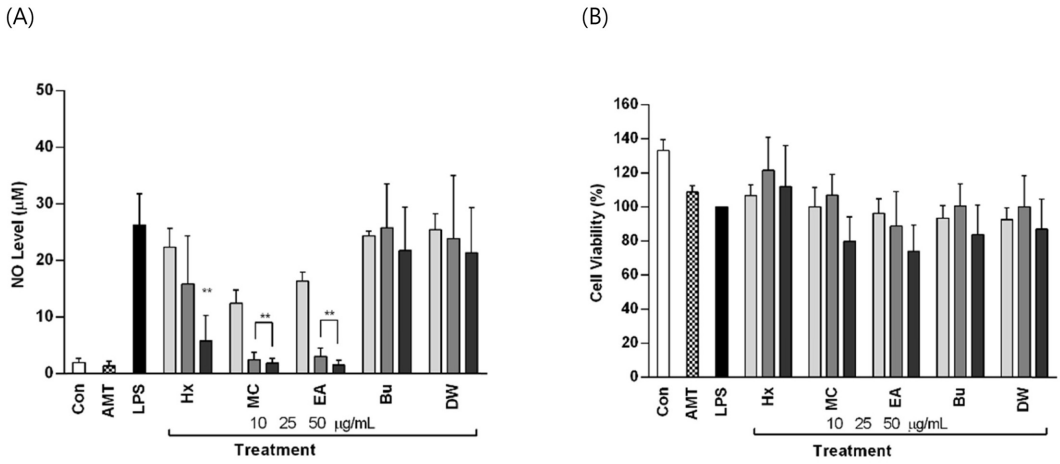

Fig. 1. Effect of Arecae Pericarpium fractions on (A) LPS-induced NO production and (B) Cell viability in RAW 264.7 cells.

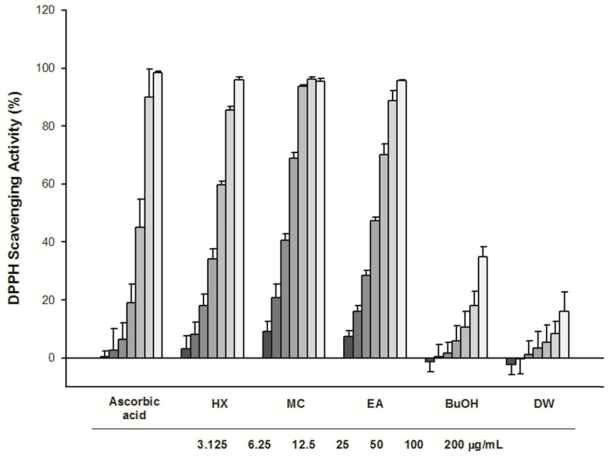

Fig. 2. DPPH scavenging activity of Arecae Pericarpium fractions.

fractions to inhibit LPS-induced NO production in RAW 264.7 macrophage cells is shown in Fig. 1. MC fraction showed the strongest inhibition with IC_{50} value 8.89 μg/mL followed by EA fraction with an IC_{50} value 13.60 μg/mL. The hexane fraction inhibited NO production with an IC_{50} value of 31.94 μg/mL. Both BuOH and DW fractions were above 50 μg/mL. MTT assay was performed to ensure that the samples in tested concentrations did not affect the cell viability which may provide false positive results on the inhibition of LPS-induced NO production. As seen from Fig. 1, EA fraction showed cytotoxicity at 50 μg/mL (cell viability was reduced to 74%) while other fractions showed an acceptable range of cell viability range (>80%).

The potential anti-oxidant activity of Arecae Pericarpium was determined by DPPH radical scavenging assay. As seen from Fig. 2, MC fraction showed the highest scavenging activity with EC_{50} value 21.30 μg/mL. The EC_{50} values of HX and EA fractions were 40.77 and 55.49 μg/mL, respectively. The EC_{50} value of ascorbic acid was 57.07 μM (or 10.05 μg/mL). BuOH and DW fractions showed weak scavenging activity with an EC_{50} value above 200 μg/mL. Arecae Pericarpium has been reported to have anti-oxidant activity, which was weaker than Arecae Semen but stronger than other parts of the same plant.[3,12] This result confirmed the anti-oxidative potential of Arecae Pericarpium in the previous studies.

The IC_{50} and EC_{50} values of Arecae Pericarpium fractions on NO production and DPPH scavenging activity are listed in Table 1.

The melanogenesis inhibition activity of Arecae Pericarpium methanol (MeOH) extract and fractions were examined with mushroom tyrosinase and melanin content assay. Mushroom tyrosinase assay is a cell-free based

Table 1. IC_{50} and EC_{50} values of Arecae Pericarpium Fractions on LPS-induced NO production and DPPH scavenging activity

Fraction	NO Production [μg/mL][a]	DPPH Scavenging [μg/mL][b]
HX	31.94	40.77
MC	8.89	21.39
EA	13.60	55.49
BuOH	>50	>200
DW	>50	>200

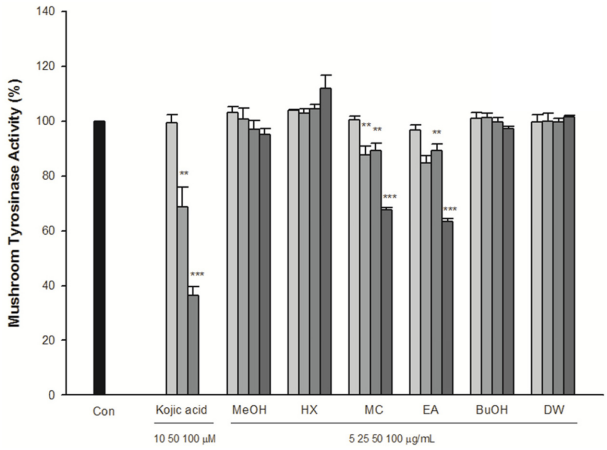

Fig. 3. Mushroom tyrosinase inhibition activity of Arecae Pericarpium fractions.

assay to determine whether the tested agents are able to inhibit tyrosinase. Tyrosinase is the rate-limiting enzyme in the melanin synthesis cascade which converts L-tyrosine to DOPA quinine, precursor of melanin.[13]

Fig. 3 showed that MC and EA fractions at 100 μg/mL were able to inhibit mushroom tyrosinase activity to 67.54% and 63.31%, respectively, while other fractions did not show any effect. Kojic acid was used as a positive control. Since MC and EA fractions showed positive results, cellular melanin content was determined to examine whether Arecae Pericarpium is able to reduce the production of melanin in melanoma cells.

B16F10 melanoma cells were stimulated with 1 mM theophylline to increase melanin production. The ability of the samples to decrease the melanin production was investigated by determining the extracellular and intracellular melanin content. MTT assay was also performed to assess the effect of tested concentrations on the cell viability. Arbutin was used as a positive control for this experiment. Fig. 4 shows that BuOH and DW fractions did not show any effect. The HX fraction reduced melanin content only at 100 μg/mL, which were to 82.34% (extracellular) and 72.78% (intracellular). MC and EA fractions which showed good LPS-induced NO inhibition,

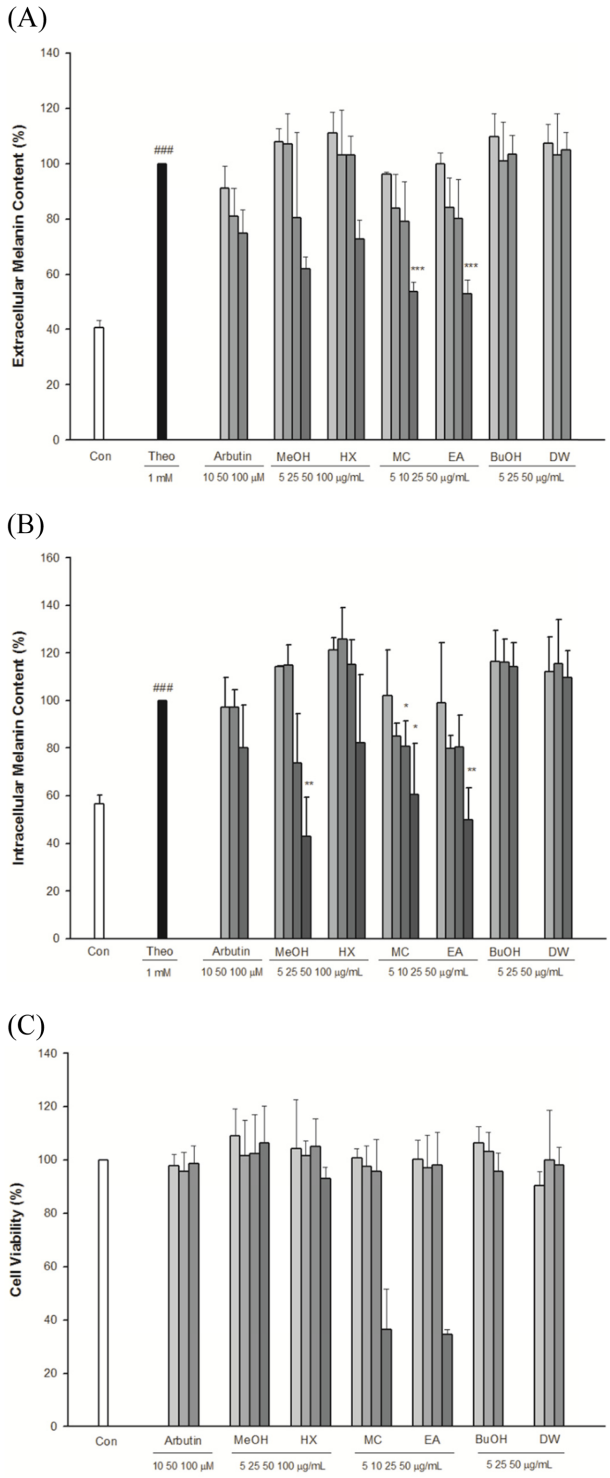

Fig. 4. Effect of Arecae Pericarpium extract and fractions on (A) Extracellular melanin content (B) Intracellular melanin content (C) Cell viability in B1F10 melanoma cells.

DPPH scavenging, and mushroom tyrosinase inhibition activity were only able to show 20% reduction of melanin content at 25 μg/mL. Cytotoxicity was shown at 50 μg/mL, thus the low percentage of melanin content was caused by the low number of living cells.

Arecae Pericarpium MeOH extract was then tested to examine whether it has melanogenesis inhibition activity and less cytotoxicity. The result showed that MeOH extract has greatest melanin content reduction. The extracellular and intracellular melanin content was reduced to 61.88% and 42.80% at 100 μg/mL, respectively, without cytotoxicity effect.

Since Arecae Pericarpium exhibited the potential biological activities, a bioassay-guided isolation was conducted to analyze the potential active compounds in Arecae Pericarpium. Griess reagent assay was used for the bioassay-guided isolation scheme. The isolation was conducted on MC and EA fractions with various chromatography techniques as described in Fig. 5 and Fig. 6, since they showed higher potential anti-inflammatory activity.

Eight known phenolic compounds were isolated from MC and EA fractions of Arecae Pericarpium. Based on the NMR results, the compounds were identified as dimeric syringol (**1**) (yield: 9.2 mg), catechol (**2**) (yield: 25.4 mg), 4-hydroxybenzaldehyde (**3**) (yield: 1.0 mg), vanillin (**4**), 4-hydroxyacetophenone (**5**) (yield of **4** and **5** mixture: 5.0 mg), apocynin (**6**) (yield: 0.6 mg), protocatechuic acid (**7**) (yield: 18.7 mg) and 4-hydroxybenzoic acid (**8**) (yield: 3.3 mg). Their structures are shown in Fig. 8.

All eight phenolic compounds were tested to compare their activity in inhibiting LPS-induced NO production. Among the isolated compounds, catechol showed the strongest inhibition of LPS-induced NO production. In addition, based on the NF-κB SEAP assay result, catechol showed NF-κB inhibition on LPS-stimulated cells. Particularly, cells treated with 20 μM catechol showed greater inhibition compared to positive control.

In the present study, the active compounds showing potential anti-inflammatory, anti-oxidant, and melanogenesis inhibition activities of Arecae Pericarpium were investigated through bioassay-guided isolation scheme. MC and EA fractions showed higher LPS-induced NO production, DPPH radical scavenging activity, and melanogenesis inhibition compared to other fractions. Both fractions also showed cytotoxicity above 25 μg/mL. When the fractions were compared to MeOH extract, the extract did not show any inhibition on tyrosinase activity but reduced the melanin content to the lowest level among all tested samples. Additionally, the MeOH extract did not cause cytotoxicity even at 100 μg/mL, while the fractions showed cytotoxicity at concentration lower than 100 μg/mL. Since MeOH extract consists of more compounds than the fractions, the synergism of compounds in the extract may exert less

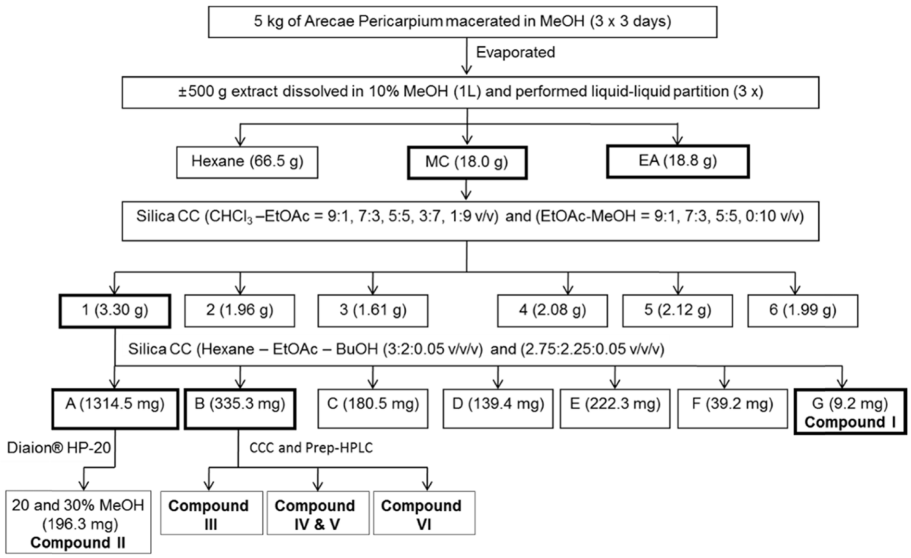

Fig. 5. Isolation scheme of Arecae Pericarpium methylene chloride fraction.

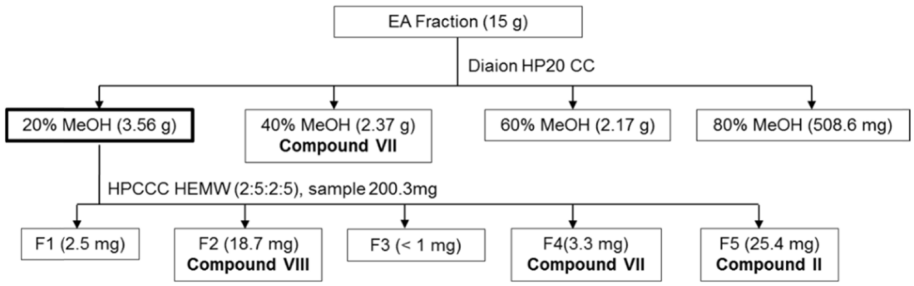

Fig. 6. Isolation scheme of Arecae Pericarpium ethyl acetate fraction.

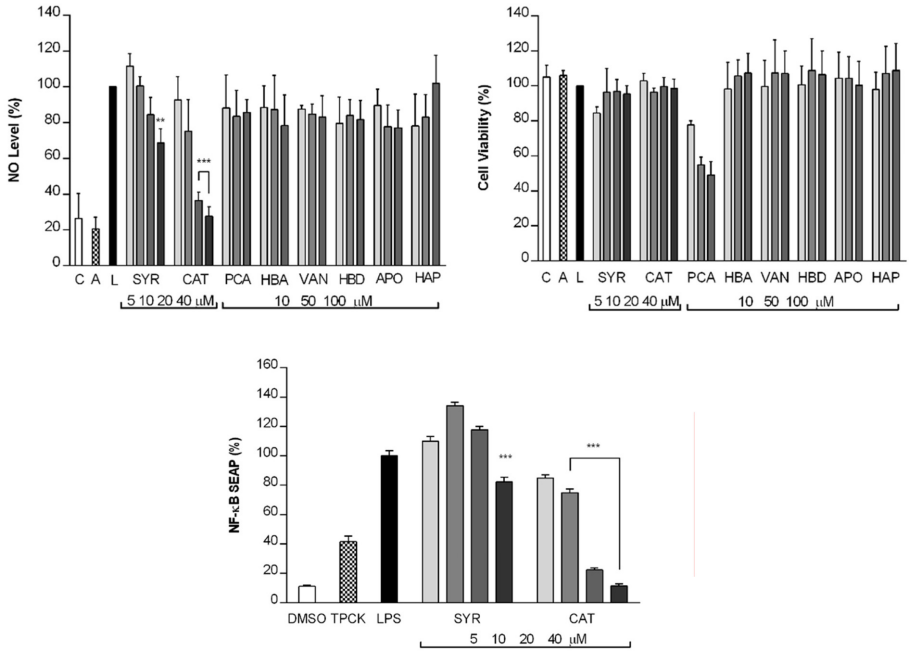

Fig. 7. Effect of isolated compounds on (A) NO production and (B) cell viability and (C) NF-κB inhibition activity of syringol and catechol.

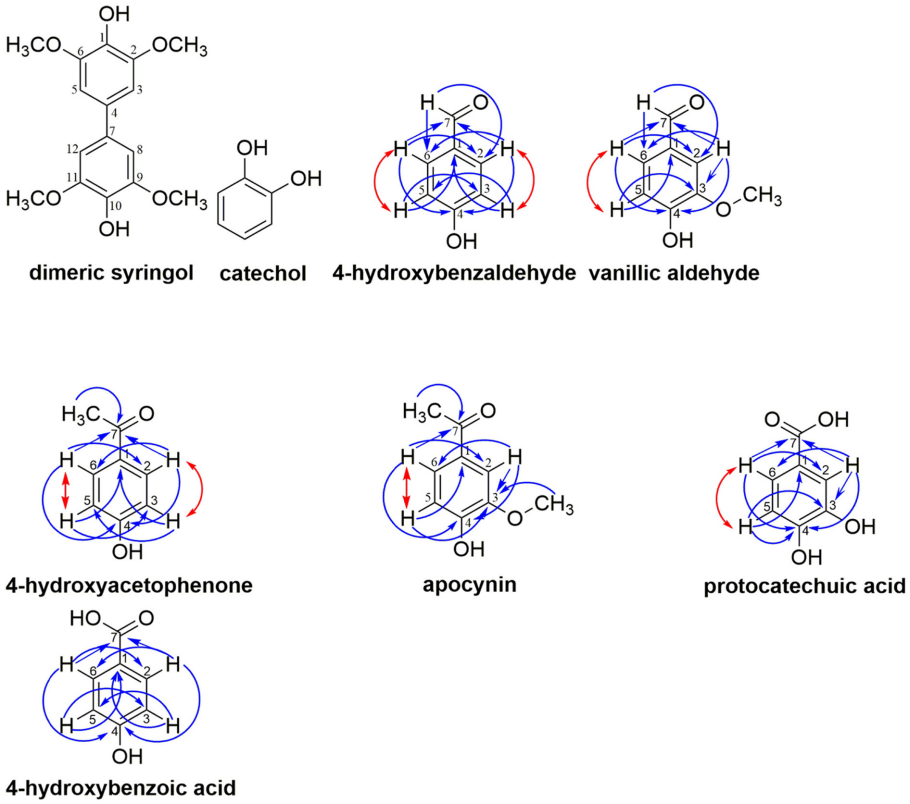

Fig. 8. Isolated phenolic compounds from Arecae Pericarpium MC and EA fractions.

negative effect on cell viability compared to the fractions. At this concentration, the intracellular melanin content was found to be lower than that of control. This result may suggest that at high concentration the MeOH extract inhibits the melanin production to an extent that it may lead to skin depigmentation. Thus, the maximum concentration use of MeOH extract for skin-whitening agent should be less than 100 μg/mL. Further studies are needed to elucidate the mechanism of Arecae Pericarpium in inhibiting melanogenesis and most importantly, to assess its safety for the use as a dermatological product.

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