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Original Research Article

Determination of Antioxidant and Anti-Melanogenesis Activities of Indonesian Lai, *Durio kutejensis* [Bombacaceae (Hassk) Becc] Fruit Extract

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Abstract

Purpose: *To investigate the antioxidant and anti-melanogenesis activities of Durio kutejensis [Bombacaceae (Hassk.) Becc] fruit extract.*
Methods: *The fruit flesh of D. kutejensis was extracted successively with n-hexane, ethyl acetate/EtOAc, and ethanol/EtOH at room temperature repeatedly. The extracts were concentrated in vacuo to yield their residues. Antioxidant properties were analyzed by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, superoxide dismutase (SOD)-like activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and oxygen radical absorbance capacity (ORAC) while anti-melanogensis activity was evaluated by tyrosinase enzyme activity and B16 melanoma cell assays (melanin inhibition and cytotoxicity).*
Results: *The extract (200 µg/mL) showed melanin inhibition by inhibiting melanin formation in B16 melanoma cell by 47 % without cytotoxicity but did not inhibit tyrosinase enzyme activity. The extract (1 - 1000 µg/mL) also exhibited some level of antioxidant activity including ORAC (0.04 ± 0.00 µmol TE/mg at 950 µg/mL), ABTS (1.0 ± 0.2 % at 100.8 µg/mL), SOD (IC50, 76.00 ± 14.6 µg/mL, and DPPH (21.5 ± 0.7 % at 97.39 µg/mL extract concentration).*
Conclusion: *The fruit extract of Durio kutejensis has antioxidant properties with a potential for treating hyperpigmentation and for use as a skin-lightening agent.*

Keywords: *Durio kutejensis, Antioxidant, Anti-melanogenesis, B16 Melanoma cell Hyperpigmentation, Skin-lightening agent*

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INTRODUCTION

Melanin is the major pigment responsible for the color of human skin. It is secreted by melanocyte cells in the basal layer of the epidermis [1]. Melanin pigments are formed in specialized pigment-producing cells known as melanocytes, which originate in the neural crest during embryogenesis and are distributed throughout the embryo during its development [2].

Melanin biosynthesis (melanogenesis) occurs in a cascade of enzymatic and spontaneous reactions that convert tyrosine to melanin pigments. The initial and rate-limiting step in melanin synthesis is the hydroxylation of tyrosine to dihydroxyphenylalanine or DOPA [3]. DOPA-oxidation produces a highly reactive intermediate that is further oxidized to form melanin by a free radical-coupling pathway. If free radicals are inappropriately processed in melanin biosynthesis, hydrogen peroxide (H_2O_2) is generated, leading to the production of hydroxyl radicals (HO•) and other reactive oxygen species or ROS [4].

Fruit is a natural product, and Indonesia's remarkable tropical biodiversity includes fruits that have provided significant contributions to national development programs such as those involving food, nutrition, drugs and pharmaceutical agents. In the present study, we evaluated the extract of *Durio kutejensis* or Lai fruit, an endemic fruit found in East Kalimantan, Indonesia for its antioxidant activities and anti-melanogenesis effect (i.e., melanin inhibition in B16 melanoma cells and mushroom tyrosinase inhibition).

EXPERIMENTAL

Reagents

Mushroom tyrosinase, trolox, MTT, fluorescein sodium salt and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The 7-methoxycoumarin was from Indofine (Hillsborough Township, NJ). EMEM was from Nissui Chemical Co. (Osaka, Japan). EDTA was from Dojindo (Kumamoto, Japan). NaOH, DMSO, L-tyrosine, miconazole, L-DOPA, 1.1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) and potassium peroxodisulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tris (hydroxymethyl) aminomethane was from Sigma-Aldrich Japan (Tokyo). Other chemicals used were of the highest commercial grade available.

Plant material and extraction

The fruit (Figure 1) was collected in January 2012 in Samarinda, East Kalimantan, Indonesia. The samples were identified by Dr. Medi Hendra, then authenticated in Bogor Herbarium Institute, Bogor, Indonesia with no. BO 1381469. The voucher specimen (F-12) and deposited in Laboratory of Physiology, Faculty of Mathemathics and Science, Mulawarman University. The fruit flesh of *D. kutejensis* (90.18 g) was extracted successively with n-hexane (400 mL), ethyl acetate/EtOAc (250 mL), and ethanol/EtOH (350 mL) at room temperature repeatedly. The extracts were concentrated in vacuo, and they gave residues of 0.412, 0.808, and 0.254 g, respectively.

Figure 1: Fruit of *D. kutejensis* (Yellow color is flesh of fruit)

SOD-like (equivalent) activity assay

SOD-like activity was measured using the SOD assay kit-WST (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. The method involved is based on previous experiments by Ukeda *et al* [5]. Trolox was used as a positive control.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay

ABTS assay was mostly based on the method described by Re *et al* [6] in which ABTS•+, the oxidant, was generated by persulfate oxidation of ABTS. Specifically, to 5 mL of 7 mM ABTS ammonium aqueous solution, 88 µL of 140 mM potassium peroxydisulfate ($K_2S_2O_8$) was added, and the resulting mixture was then allowed to stand at room temperature for 12 - 16 h, yielding a dark blue solution. The mixture was then adjusted by 99.5 % ethanol so that it gave an absorbance of 0.7 ± 0.02 units at 734 nm (UVmini-1240, Shimadzu, Kyoto, Japan) to make the working solution. One milliliter of working solution was mixed with 10 µL of extract dissolved in ethanol and shaken well for 10 s; after 4 min of incubation at 30 °C, the absorbance of the reaction mixture was measured at 734 nm. Trolox was used as positive control.

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH assay was performed as described by Arung *et al* [7]. In brief, the sample was first dissolved in DMSO and used for the actual experiment at 30 times dilution. The reaction mixture contained 967 µL of 60 mM DPPH in ethanol and 33 µL of sample solution in DMSO. After the reaction was carried out at room temperature for 30 min, the free radical scavenging activity of the sample was quantified by the decolorization of DPPH at 514 nm.

Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was performed as described by Ou *et al* [8] with some modification modification. Briefly, 300 µL of the standard (Trolox) or sample solution is mixed with 1.8 mL of 48 nM FL solution, and then they were incubated independently at 37 ˚C for 15 min. AAPH solution (900 µL; 12.9 mM, final concentration) was added to the mixture and vortexed for 10 s. Then, it immediately placed in a fluorescence spectrophotometer (Model FP-6500, JASCO Co, Ltd, Tokyo, Japan), and measured every 5 s for 60 min at 37 ˚C (Ex: 485 nm, Em: 520 nm). A blank (FL + AAPH) using phosphate buffer, standard solutions (6.25-50 µM Trolox), and sample solutions were measured at same conditions. Three independent assays were performed for each sample. The area under the fluorescence decay curve (AUC) was calculated using Eq 1.

$$AUC = 1 + \{\Sigma(fi/fo)\} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where fo is the initial fluorescence reading at 0 min and fi is the fluorescence reading at time, i.

The ORAC values were calculated as Prior *et al* [9] by using a equation (Y) a + b(X) between Trolox concentration (Y) (µM) and the net area under the FL decay curve (X). Linear regression was used in the range of 6.25-50 µM Trolox. Data are expressed as micromoles of Trolox equivalents (TE) per micromole of sample (µmol TE/µmol).

Tyrosinase assay

Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used for the present experiment due to its ready availability. It should be noted that commercial tyrosinase was reported to contain numerous proteins in addition to tyrosinase [10] but it was used without purification. In the present study, we determined the tyrosinase activity as described [7]. Kojic acid was used as positive control.

Cell culture

A mouse melanoma cell line, B16, was obtained from the RIKEN Cell Bank (Ibaraki, Japan). The cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS) and 0.09 mg/mL theophylline. The cells were incubated at 37 ^{o}C in a humidified atmosphere of 5 % CO_2. This assay was determined as described by Arung *et al* [7] as seen below.

Determination of melanin content in B16 melanoma cells

The melanin content of the cells after treatment was determined as follows. After we removed the medium and washed the cells with PBS, the cell pellet was dissolved in 1.0 mL of 1 N NaOH. The crude cell extracts were assayed using a microplate reader (Bio-Tek, Winooski, VT) at 405 nm to determine the melanin content. We analyzed the results from the cells treated with the test samples as a percentage of the results from the control culture. Arbutin was used as a positive control.

Determination of cell viability

We determined the cell viability by the micro-culture tetrazolium technique (MTT). MTT assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells. The cultures were initiated in 24-well plates at 1×10^5 cells per well. After incubation, we added 50 µL of MTT reagent [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide in PBS (5 mg/mL)] to each well. The plates were incubated in a humidified atmosphere of 5 % of CO_2 at 37 ^{o}C for 4 h. After removing the medium, we added 1.0 mL isopropyl alcohol (containing 0.04 N HCl) to the plate, and the absorbance was measured at 570 nm relative to 630 nm.

Statistical analysis

The IC_{50} (median inhibition concentration) is the concentration of the compound that reduces the biological activity by 50 % and determined by JMP Pro ver. 9.0.2 (SAS Institute Japan Ltd). Value was given as geometric means. Differences were considered to be statistically significant when $p < 0.05$ and < 0.01.

RESULTS

Antioxidant effect of the extract

The ethyl acetate extract of *D. kutejensis* fruit was more potent as an antioxidant than other extracts. It showed some antioxidant activity in ABTS, SOD-like activity, DPPH, and ORAC assays, as follows: 1.0 ± 0.2 % at 100.8 µg/mL, 76.5 ± 14.6 (IC_{50} µg/mL), 21.5 ± 0.7 % at 97.4 µg/mL, 0.04 ± 0.0 TEAC (µmol TE/mg), respectively (Table 1). Trolox was used as positive control.

Anti-melanogenesis effect of the extract of *D. kutejensis*

The tyrosinase enzyme activity of the extract is presented in Table 2, with L-DOPA the as substrate. The extracts did not show tyrosinase inhibition activity, compared with Kojic acid as positive control which inhibited tyrosinase activity.

In the evaluation of melanin inhibition in the B16 melanoma cells, the EtOAc extract of *D. kutejensis* fruit showed more poten than others in melanin inhibition of 46.9 % with no cytotoxicity at 200 µg/ml) as shown in Table 2. Arbutin, used as positive control showed 62.4 % of inhibition with less toxicity.

DISCUSSION

Several fruits have been found to have potency as a cosmetic material. Examples include citrus fruits, which contain hesperetin, induce melanogenesis [11]; *Cucumis sativus* has anti-hyaluronidase activity [12]; *Embelica officinalis* has an anti-photoaging effect [13], and *Platycarya strobilacea* has an anti-wrinkle effect [14]. In Indonesia, the fruit of Langsat (*Lansium domesticum*) from Central Kalimantan is produced for a cosmetic product offered by the Martha Tilaar Company. The fruit of Limpasu (*Baucarea* sp.) is produced traditionally in small-scale industrial outfits in South Kalimantan, Indonesia, as Bedak Dingin.

The skin is a major target of oxidative stress caused by reactive species (RS), including reactive oxygen species and reactive nitrogen species. RS is major and significant contributors to skin hyperpigmentation and skin aging [15]. It has generally been believed that agents that have antioxidant activity show anti-aging, whitening, and anti-inflammatory activities [16]. Here we conducted antioxidant assays (ABTS, SOD-like activity, DPPH and ORAC) to determine the ability of the fruit extract to counteract oxidative stress from UV irradiation. The EtOAc extract showed promise as an antioxidant. To the best of our knowledge, this is

Table 1: Effect of Lai (*Durio kutejensis*) fruit extracts on antioxidant assays

Samples	ABTS IC_{50} (µg/ml)	SOD like activity IC_{50} (µg/ml)	DPPH IC_{50} (µg/ml)	ORAC TEAC (µmol TE/mg)
n-Hexane	2.6 ± 0.5 % (at 106.8 µg/ml)	18.2 ± 1.0 % (at 106.8 µg/ml)	nt	nt
EtOAC	1.0 ± 0.2 % (at 100.8 µg/ml)	76.5 ± 14.6	21.5 ± 0.7 % (at 97.4 µg/ml)	0.04 ± 0.00
EtOH	2.4 ± 0.4 % (at 101.2 µg/ml)	30.2 ± 9.8 %(at 101.2 µg/ml)	nt	nt
Trolox	30.6 ± 0.8	246.9 ± 14.1		

nt: not tested. Each column represents the mean±SD, with n = 3. The IC_{50} was determined by JMP Pro ver. 9.0.2

Table 2: Anti-melanogenesis effect of *D. kutejensis* fruit extracts (tyrosinase assay and melanin inhibition in B16 melanoma cells)

Test agent	L-DOPA[a] (% inhibition)	Melanin inhibition (µg/ml)	Cell viability (%)
n-Hexane	No inhibition[b]	19.7 (at 200 µg/ml)**	89.9**
EtOAC	No inhibition[b]	46.9 (at 200 µg/ml)**	101.2*
EtOH	05.00 (at 100 µg/ml)*	15.5 (at 200 µg/ml)*	99.7
Kojic acid	87.9 (at 10 µg/ml)**	-	-
Arbutin	-	62.4 (at 100 µg/ml)**	96.2**

Note: *a = substrate; b = at 100 µg/ml; each column represents the mean ± SD, n = 3); p < 0.05 (*), p < 0.01 (**), compared with control; DOPA = 3,4-dihydroxyphenylalanine*

the first report that this fruit extract has the ability to inhibit melanin in B16 melanoma cells along with some antioxidant activities.

Initially, we examined all extracts to tyrosinase inhibitory activity, but no inhibition. Next, we done with B16 melanoma cells to evaluate its melanin inhibition and the EtOAc extract inhibited the melanin production in B16 melanoma cells. Some active compounds might be involved inhibit melanin production in B16 melanoma cells. Rudiyansyah and Garson were reported that wood bark of *Durio kutejensis* contained triterpenes (3β-O-trans-caffeoyl-2α-hydroxy-olean-12-en-28-oic acid, 3β-O-trans-caffeoyl-2 α -hydroxytaraxest-12-en-28-oic acid, maslinic acid, and arjunolic acid), quinone (2,6-dimethoxy-p-benzoquinone), and coumarin (fraxidin) [17]. The active compounds of triterpene, quinone and coumarin were reported as melanin inhibitor [18-20]. The extract might not have directly inhibited tyrosinase enzyme but probably acted via signal transduction of melanogenesis in the cells, leading to inhibition of melanin formation. Lam *et al* stated that some signal transductors such as cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), tyrosinase-related protein (TRP), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and microphthalmia associated transcription factor (MITF) play some role in the mechanism of anti-hyperpigmention [21]. However, this hypothesis needs to be tested in further studies. To the best of our knowledge, this is the first report that this fruit extract of Lai has the ability to inhibit melanin in B16 melanoma cells along with some antioxidant activities.

CONCLUSION

The EtOAc extract of *D. kutejensis* fruit exhibits melanin inhibition with no cytotoxicity, as well as antioxidant activity. Thus, this extract may have potentials for treatment of hyperpigmentation as well as an application skin-lightening agent. However, further studies are necessary to isolate the active compound, evaluate its mechanism of action, as well as ascertain its safety profile.

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