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Research article

Antimelanogenesis and cellular antioxidant activities of rubber (*Hevea brasiliensis*) seed oil for cosmetics

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ABSTRACT

The abundant rubber seed, the by-product of rubber tree (*Hevea brasiliensis*), is used for tree plantation, biodiesel production, coating, and animal feed. The reported major unsaturated fatty acid components of rubber seed oil are involved in the biological activities and may potentially be employed in the cosmetics. Nevertheless, the biological activity study of rubber seed oil for cosmetic application is limited. The aims of study were to extract the rubber seed oil, analyze the fatty acid components, and evaluate for the cytotoxicity by sulforhodamine B assay and biological activities, including melanogenesis assay and antioxidant activity, in cell culture. The extraction yield of rubber seed oil was 19.32 ± 0.54%. The fatty acid components of rubber seed oil were palmitic (18.90 ± 0.55%), stearic (10.91 ± 0.25%), oleic (35.91 ± 1.97%), and linoleic (33.31 ± 1.56%) acids. The non-cytotoxic concentrations of rubber seed oil at 0.0001-0.1 mg/mL were shown the cell viability higher than 80% in B16-F10 melanoma cells and 3T3-L1 cells. The concentration of rubber seed oil that inhibited cell viability at 50% (IC_{50}) was 0.29 mg/mL in B16-F10 melanoma cells. The melanogenesis assay of rubber seed oil at 0.1 mg/mL was shown the inhibitory effect on melanin content (46.24 ± 2.64%), tyrosinase activity (53.85 ± 5.04%) and tyrosinase-related protein-2 activity (66.01 ± 1.84%). The antioxidant activity of rubber seed oil was demonstrated the cellular protective effect at 0.001 mg/mL with cell viability of 99.72 ± 6.92%. The results of study have been supported the potential utilization of rubber seed oil as a functional raw material to be incorporated into the personal care and cosmetic preparations.

1. Introduction

Rubber tree (*Hevea brasiliensis*), a plant from South America, is an industrial tree cultivated worldwide in a total area of about 10 million hectares to harvest for production of latex. The main regions of rubber tree plantations are in Southeast Asia, particularly in Indonesia, Malaysia and Thailand (Gerber, 2011; Mohd-Setapar et al., 2013). Since the rubber latex is in high demand for many industries, including the tire industry, rubber glove industry and medical device industry, the prices of rubber do not fall during the last 50 years (Gerber, 2011) and the expanded monoculture rubber tree plantations have been reported over the past two decades (Yi et al., 2014). Due to the market supply for rubber latex is increased, the by-product obtained from rubber tree is abundant, particularly rubber seeds. The rubber seed is normally used for tree plantation and some studies have been demonstrated on the application in biodiesel production, non-polluting and environmental friendly coating, and animal feed (Aigbodion and Pillai, 2000; Babatunde et al., 1990; Ikwuagwu et al., 2000). The main fatty acid components of rubber seed oil are the unsaturated fatty acids, including oleic acid, linoleic acid and linolenic acid, which have been reported to exhibit the biological activities (Aigbodion and Pillai, 2000; Ando et al., 1998; Lourith et al., 2014; Manosroi et al., 2010) and may potentially be employed as a functional raw material for personal care and cosmetic preparations. Nevertheless, the biological activity study of rubber seed oil for application in cosmetics is limited.

Nowadays, the natural ingredients have been increasingly interested to incorporate in many personal care and cosmetic products. The consideration on health, environmental awareness and safety of synthetic chemicals are the factors driven for manufacturing of natural origin products. For the botanical ingredients in personal care and cosmetic products, the plant oils are used as the functional ingredients, including antioxidant, anti-carcinogen, and photo-protector (Antignac et al., 2011). Fatty acids, which are in combination with glycerol to form the major compositions of plant oils, are responsible for the characteristics and biological activity of each plant oil (Montero de Espinosa and Meier, 2011).

In this study, the rubber seed oil was extracted by a Soxhlet apparatus and investigated for the fatty acid profile by gas chromatography-

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mass spectrometry (GC/MS). The cytotoxicity assay and biological activities related to cosmetic application, including melanogenesis assay and antioxidant activity, were performed in cell culture in compared to the standard compounds used in the cosmetic products and the fatty acid components of rubber seed oil.

2. Materials and methods

2.1. Chemicals and reagents

Hexane, sodium hydroxide, dichloromethane, methanol, toluene, hydrochloric acid, and ethanol were analytical grade from RCI Labscan Limited (Bangkok, Thailand). *L*-Ascorbic acid (vitamin C), sulforhodamine B (SRB), kojic acid, theophylline, mushroom tyrosinase, and melanin were purchased from Sigma Aldrich Co. (Missouri, USA). Dulbecco's modified Eagle medium (DMEM) and penicillin/streptomycin solution were from Gibco (Maryland, USA). Fetal bovine serum was from PAA Laboratories GmbH (Pasching, Austria). The other reagents were of analytical grade.

2.2. Preparation of rubber seed oil

The rubber seed of RRIM 600 cultivar was harvested from the plant field at Sisaket province in the Northeastern region of Thailand and extracted as previously reported (Lourith et al., 2014). Briefly, the seed was dried in hot air oven at 45 °C, ground into powder, and kept at −20 °C until extraction. The seed powder was extracted by using a Soxhlet extractor with *n*-hexane for 6 h. The ratio of seed powder and solvent was 25 g per 750 mL. The extraction was undertaken for 3 times and calculated for the extraction yield.

2.3. Analysis of fatty acid profile

The mild esterification of rubber seed oil was prepared as previous report (Ichihara and Fukubayashi, 2010). Briefly, the rubber seed oil was mixed with toluene, methanol, and 8% hydrochloric acid. The mixture was incubated at 45 °C for 24 h, partitioned with *n*-hexane, dried over with magnesium sulfate anhydrous, and concentrated to dryness in vacuo. An aliquot (1 μL) of sample diluted (1:1, v/v) with dichloromethane was injected (220 °C) in the splitless mode into a gas chromatograph (Agilent, 6890 N, USA) equipped with a HP-5MS capillary column (Agilent, 30 m × 250 μm, 0.25 μm film thickness) and mass spectrophotometer (Agilent, 5973N). The analysis was used helium as the carrier gas (1 mL/min). The separation was performed on the program started from 50 °C (5 min), rising to 65 °C at a rate of 2 °C/min, and then 200 °C (5 °C/min, 5 min) and 250 °C (10 °C/min) that was held for 10 min. The retention time and mass spectra of compounds were identified using the Wiley 7n.1 database. The analysis of rubber seed oil components by esterification was performed in triplicate.

2.4. Cytotoxicity assay

2.4.1. Cell culture

B16-F10 melanoma cells (ATCC® CRL-6475™) and 3T3-L1 cells (ATCC® CL-173™) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution at 37 °C in a humidified incubator with 5% CO_2 (Binder, model CB210, Germany). Cells were grown to 70–75% confluence and harvested to count by using hemocytometer. All experiments in cell culture were performed in triplicate.

2.4.2. Sample preparation for cytotoxicity and biological activitiy assays in cell culture

The rubber seed oil and the fatty acid components of rubber seed oil, including palmitic, stearic, oleic and linoleic acids, were dissolved in absolute ethanol and sterilized by filtration through 0.2 μm membrane. Theophylline, kojic acid, and vitamin C were dissolved in the culture medium and then filtered for sterilization.

2.4.3. Cytotoxicity assay by SRB assay

Cytotoxicity assay was determined by the SRB assay (Papazisis et al., 1997). Briefly, B16-F10 melanoma cells or 3T3-L1 cells were seeded at the density of 1×10^4 cells/well in 96-well plates and incubated overnight for cell adhesion. Cells were then treated with various concentrations (0.0001–1 mg/mL) of rubber seed oil, fatty acid components of rubber seed oil, vitamin C, and the solvent. The final concentration of ethanol used as a control solvent was less than 1%. After 72-h incubation, the treated cells were fixed with 50% trichloroacetic acid, washed and dyed with SRB. The bound dye was solubilized with 10 mM tris buffer and the absorbance was measured at 540 nm using a microplate reader. The percentage of cell viability was calculated according to the following equation:

$$\% \text{ Cell viability} = (A/B) \times 100$$

Where, A was the absorbance of sample and B was the absorbance of control (solvent).

2.5. Melanogenesis assay in B16-F10 melanoma cells

2.5.1. Melanin content

The melanin content was measured according to the previously described method with slight modification (Oka et al., 1996). Briefly, B16-F10 melanoma cells at the density of 1×10^5 cells/well were plated in 6-well plates and incubated overnight for cell adhesion. The samples were then added and incubated for 72 h. The cells were washed and dissolved in 500 μL of 2 M sodium hydroxide at 60 °C for 1 h. The absorbance was measured at 450 nm using a microplate reader and the melanin content was compared to the standard melanin. The total protein content of cell lysate was also evaluated by the Bradford dye-binding reagent using bovine serum albumin as a standard and measured the absorption at 595 nm (Bradford, 1976). For the determination of the actual melanin formation from the same cell concentration, the melanin content of each treatment was divided by the total protein content. The percentage of relative ratio of melanin content was calculated as the following equation:

$$\% \text{ Relative ratio of melanin content} = (Mt/Mc) \times 100$$

Where, Mt was the melanin content of sample divided by the total protein content of sample and Mc was the melanin content of control divided by the total protein content of control (solvent).

2.5.2. Tyrosinase activity

Tyrosinase activity was analyzed by the method described by Ohguchi et al. with slight modification (Ohguchi et al., 2005). Briefly, the sample treated B16-F10 melanoma cells were washed with ice-cold phosphate buffer saline solution and then lysed by the lysis reagent containing protease inhibitor at 4 °C for 30 min. The lysates were centrifuged at 15,000 rpm for 10 min. The obtained supernatant was collected, mixed with the mixture containing 50 mM sodium phosphate buffer (pH 6.8) and 0.05% dopa, and further incubated at 37 °C for 2 h. After incubation, the dopachrome formation was measured at 490 nm using a microplate reader. The enzyme activity was calculated in compared to the standard mushroom tyrosinase. The total protein content of sample was also evaluated. Enzyme activity of the sample was compared to the control and calculated as the percentage of relative ratio of tyrosinase activity as shown in the following equation:

$$\% \text{ Relative ratio of tyrosinase activity} = (Tt/Tc) \times 100$$

Where, Tt was the tyrosinase activity of sample divided by the total protein content of sample and Tc was the tyrosinase activity of control divided by the total protein content of control (solvent).