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Original Article

Antioxidative and melanin production inhibitory effects of *Syzygium cumini* extracts

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Abstract

Syzygium cumini (L.) Skeels is a famous herbal tree in Myrtaceae family. Antioxidative activity of its ethanolic extract was determined using DPPH assay and the result showed that *S. cumini* leaf extract (SLE) exhibited stronger antioxidative properties than its branch extract (SBE) with IC_{50} values at 4.58±0.07 and 5.80±0.03 µg/mL, respectively. Moreover, both SLE and SBE revealed high total phenolic contents which were related to their antioxidative activities. In addition, inhibition of melanin production was assessed in B16-F10 murine melanoma cells. After 48 hr of incubation at concentrations between 0.06-1.00 mg/mL, cells exhibited no toxicity and inhibited melanin production. RT-PCR analysis indicated the extracts inhibited the expression of tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2 genes in a dose-dependent manner. The findings reveal the antioxidative and melanin production inhibitory properties of the *S. cumini* extracts. In particular, SLE should be further developed for skin whitening products.

Keywords: *Syzygium cumini*, tyrosinase, melanin, B16-F10 cells

1. Introduction

The differences in skin color of humans are due to the amount of the melanin pigment. Melanin is produced in epidermal melanocytes and by melanogenesis, which is stimulated by three major enzymes including tyrosinase, and tyrosinase-related proteins (TRP)-1, and TRP-2 (Yang *et al.*, 2006). Melanogenesis consists of several stages including melanin production, melanin transportation and melanosome release to keratinocytes (Harata, Okajima, Arai, Kurihara, & Nakagata, 2007). Several steps of this process can be controlled, such as inhibition of tyrosinase gene expression or tyrosinase enzyme activity (Peng *et al.*, 2013). Tyrosinase is a rate-limiting enzyme in melanin biosynthesis, catalyzing the hydroxylation of tyrosine to form 3,4-dihydroxy-phynylalanine (DOPA) and oxidation of DOPA to produce DOPA-quinone. TRP-2 acts as a DOPAchrometautomerase and catalyzes the rearrangement of DOPA-chrome to form 5, 6-dihydroxyindole-2-carboxylic acid (DHICA), while TRP-1 oxidizes DHIDA to produce carboxylated indole-quinone (Matsuyama *et al.*, 2009; Sato, Tsukimoto, Shimura, Awaya, & Kojima, 2011). Thus, TRP-1 and TRP-2 also function in the biosynthesis of melanin downstream of tyrosinase.

Melanin production depends on multiple factors including race, drug administration, inflammation, and hormone action, but most importantly exposure to both types of ultraviolet radiation (UV): UV-A and UV-B (Costin & Hearing,

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2007). UV-A radiation has a long wavelength with low energy and directly stimulates melanin production in melanocytes. In comparison, UV-B has a short wavelength with high energy, and stimulates the coordination of melanocytes and keratinocytes which also can cause skin darkness. Upon stimulation by UV, keratinocytes release melanin-producing factors including alpha-melanocyte stimulating hormone (α-MSH), prostaglandin E2 (PEG2), plasmin, adenocorticotropic hormone (ACTH) and endothelial-1. α-MSH, a naturally endogenous peptide hormone, promotes the expression of microphthalmia-associated transcription factor (MITF) which is a key regulator of the development of melanocytes as it regulates the expression of tyrosinase and tyrosinase-related protein (Choi, Ahn, Lee, Chang, & Hwang, 2005). MITF can also change fibroblasts to be melanocytes-like cells (Kim *et al.*, 2006; Qiao *et al.*, 2012). Apart from its hormone stimulating properties, UV irradiation can also produce reactive oxygen species (ROS) in the skin that may induce melanogenesis (Muddathir, Yamauchi, Batubara, Mohieldin, & Mitsunaga, 2017).

The interest in anti-tyrosinase enzyme agents as a whitening product is increasing, with a focus on naturally derived agents due to negative side effects of some synthetic compounds. Therefore, several chemicals from plant origin have been tested for cosmetic and pharmaceutical use, aimed at preventing overproduction of melanin in epidermal layers or as whitening agents. Some known natural compounds, such as kojic acid, salicylhydroxamic acid, catechins, and hydroquinone, have shown significant tyrosinase inhibition properties (Baurin, Arnoult, Scior, Do, & Bernard, 2002). However, they are not without negative side effects; compounds such as hydroquinone and kojic acid have been shown to cause cytotoxicity, skin inflammation and cancer (Chung *et al.*, 2012).

In this study, we have selected *Syzygium cumini* (L.) Skeels, a medicinal plant in the Myrtaceae family. In Thailand, it is called Wa and its wood is generally used in construction, furniture and agricultural tools, its bark has been used for anti-dysentery, anti-diarrhea, and anti-inflammation purposes. Its sweet and edible fruit has also been used as a fever-lowering agent. In addition, the phytochemical report for the leaves indicates many bioactive compounds including acylated flavonol glycosides, quercetin, myricetin, triterpenoids, esterase, galloyl carboxylase and tannin. Likewise, the branches were found to contain many compounds such as betulinic acid, friedelin, epi-friedelanol, β-sitosterol, quercetin kaempferol, myricetin, gallic acid and ellagic acid (Ayyanar & Subash-Babu, 2012). Some of the above chemical contents have shown antioxidant and melanin production inhibition properties (Sripanidkulchai & Junlatat, 2014). Therefore, this study examined the antioxidant and melanin production inhibitory effects of *S. cumini*, focusing on extracts derived from its leaves and branches. The data obtained from this study are important for further pharmaceutical utilization of this plant.

2. Materials and Methods

2.1 Reagents

Molecular biology grade agarose was obtained from Bio-Rad (Spain). 1kb DNA ladder and Blue/ Orange 6X loading dye were sourced from Promega (USA). Primer β-actin, tyrosinase, TRP-1, TRP-2 were obtained from Eurofins MWG Operon (Germany). Tris base and ethylenediaminetetraacetic acid were products of Ajax (Australia). 3-[4, 5- dimethylthiazol-2-yl] -2,5-dyphenyl tetra-zolium bromide, Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were products of Invitrogen (UK). Melanin, kojic acid, tyrosinase enzyme, 2,2-diphenyl-1-picrylhydrazyl and folin phenol were products of Sigma (USA). DNase Set, Omiscript RT Kit, TopTaq Master Mix kits were received from QIAGEN (Germany). All other chemicals including Novel Juice and RNA extraction kit were obtained from GeneDirex and GE Healthcare (UK), respectively.

2.2 Preparation of the plant extract

The mature leaf and 5-6 cm diameter branches of *Syzygium cumini* were collected from Ubon Ratchathani Zoo, Ubon Ratchathani Province, Thailand. The plant was identified and authenticated by Somkao, P (taxonomist, lecturer on Thai pharmacy from Faculty of Thai Traditional and Alternative Medicine). A voucher specimen (CTAM-155) was deposited at the Faculty of Thai Traditional and Alternative Medicine, Ubon Ratchathani Rajabhat University. The samples were pulverized and macerated in 50% ethanol for 5 days, then filtered through thin cloth and centrifuged at 3000 g, 25°C for 10 min using laboratory centrifuge (Kubota, Japan). The clear supernatant was concentrated using a rotary evaporator (ETERA, Japan) at 45-50°C, then freeze-dried (Christ, Germany). The ethanolic extracts of *S. cumini* were obtained, with % yields of 8.73 and 5.25 called SLE and SBE from the leaf and branch, respectively (Table 1).

Table 1. Extraction yield, antioxidative activity and total phenolic contents of SBE and SLE

Sample	Yield (%)	DPPH* (IC_{50}, μg/mL)	Total phenolic* (eq.gallic acid), mg/g
SBE	5.25%	5.80±0.03	217.58±9.13
SLE	8.73%	4.58±0.07	341.23±11.25
Vitamin C	-	3.21±0.10	-

* Values were expressed as mean±SD (n=3)

2.3 Determination of antioxidant activity

Antioxidant activity of the plant extract was evaluated using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Briefly, the reaction was performed in 96 - well plates with the extract dissolved in 50% ethanol to various concentrations, then extracts solutions mixed with 20 μl of DPPH solution (1 mM in methanol) and incubated at room temperature in the dark for 30 min. The absorbance was measured at 515 nm using a UV spectrophotometer (Shimadzu, Japan). The DPPH solution alone in methanol was used as a control. L-ascorbic acid (Vit.C), a positive control for the DPPH method, was used as a standard antioxidant. DPPH scavenging activity was calculated using the following equation:

$$\% \text{ scavenging activity} = [(A_{control} - A_{extract})/ A_{control}]*100.$$

The percentage of scavenging activity was plotted against concentration, half maximal inhibitory concentration

(IC_{50}) of the extracts and Vit.C were calculated using linear regression analysis from the graph.

2.4 Determination of total phenolic content

The total phenolics content was determined by the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999; Sripanidkulchai & Fangkrathok, 2014). The reaction was performed in 96 – well plates with the sample solution mixed with 25 µl of the 1N Folin-Ciocalteau reagent and 70 µl of 20% sodium carbonate**.** After mixing and standing for 40 minutes at room temperature, the optical density was measured at 725 nm**.** The total phenolic contents were expressed as mg gallic acid equivalent (GAE)/g dry basis**.**

2.5 Tyrosinase inhibition assay

The method as described by Momtaz, Mapunya, Houghton, Edgerly, and Hussein (2008) was slightly modified. Briefly, the extract was dissolved in DMSO, then diluted with 50 mM phosphate buffer (pH 6.5) to different concentrations. Kojic acid was used as a positive control. 70 µl of test sample was added to a 96-well micro plate containing 100 µl of a substrate (2 mM L-tyrosine), then 30 µl of mushroom tyrosinase (167unit/mL) was added and mixed well. After incubation at room temperature for 30 min, the absorbance was determined at 492 nm with the microplate reader. The inhibitory percentage on tyrosinase activity was plotted with sample concentration, from which the half maximal inhibitory concentration (IC_{50}) value was obtained.

2.6 Cell culture and cell viability assay

B16-F10 murine melanoma cells (B16-F10 cells) were purchased from ATCC (JR scientific U.S.A.). B16-F10 cells were cultured in DMEM containing 10**%** heat-inactivated FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C, 5%CO_2**.**

The viability assay of plant extract on B16-F10 cells was determined using MTT assay (Mosmann, 1983). Cells were seeded in a 96-well plate at a density of 1 x 10^5 cells per well and allowed to attach for 24 h**.** Various concentrations of the samples were then added and incubated for 24 and 48 hr**.** After incubation, the MTT solution was added to each well at a final concentration of 0**.**1 mg/mL, and incubated for 3 h**.** The solution was removed and dimethyl sulfoxide was added**.** Finally, the cell proliferation was estimated in terms of absorbance at 570 nm**.**

2.7 Determination of melanin content

The determination of melanin content was slightly modified from a previously described method (Sato *et al.*, 2011). B16-F10 cells were seeded in 12-well plate at a density of 1x10^5 cells per well and incubated overnight, followed by addition of plant extracts**.** After 48 h of incubation, the cells were trypsinized with 0**.**25% trypsin and washed with DMEM media**.** After centrifugation, the cell pellets were solubilized in 100 µl of 1M NaOH at 60°C for 1 h**.** The melanin content was determined at the absorbance of 405 nm and compared with standard curve of melanin.

2.8 Determination of gene expression

The B16-F10 cells were plated at a density of 1x10^6 cells / well in 12-well culture plates overnight. The cells were treated with plant extracts or kojic acid then incubated at 37°C in 5% CO_2 for 48 h**.** Total RNA was isolated from the treated cells using a GE Healthcare extraction kit. cDNA was synthesized from 40 ng of total RNA with two step RT-PCR kit using a thermal cycler. Specific oligonucleotides based on the published articles were used as follows; β-actin (285 bp), FW: TCATGAAGTGTGACGTTGACATCCGT and RW: CCTAGAAGCATTTGCGGTGCACGATG; tyrosinase (386 bp), FW: ACAGAGGAGAACATCTGCCAGCTT and RW: TGGTGACTCAACAGGTGTGAAGGT; TRP-1 (428 bp), FW: ATACTGGGACCACATGGCAACACA and RW: ATTGGTCCACCCTCAGTGCTGTTA; TRP-2 (366 bp), FW: AGACTACGTGATCACCACGCAACA and RW: TTCCGACTAATCAGCGTTGGGTCA. The amplification conditions used for β-actin was 94˚C 1 min, 60˚C 1 min, and 72˚C 1 min (Won *et al.*, 2006) and 95˚C 30 min, 60˚C 30 min, and 72˚C 1 min for tyrosinase, TRP-1 and TRP-2 (Sato *et al.*, 2011). The PCR products were then analyzed on 2% agarose gel, visualized by Novel Juice staining and RT-PCR product density was measured by Gel Documentation and system analysis machine. The melanogenesis-related gene expression was expressed as the relative mRNA expression level with β-actin. Moreover, the concentration of extract to inhibit the expression of a gene at 50% (IC_{50}) was calculated by comparing with gene expression level of untreated cells (100%).

2.9 Statistic analysis

All experiments were performed in triplicate and the results expressed as mean±S.D. One-Way ANOVA and multiple comparisons were used to analyze the significant difference (P<0.05) using SPSS version 19.0 software.

3. Results and Discussion

The results revealed both ethanolic extracts of *Syzygium cumini* branch (SBE) and leaf (SLE) showed strong antioxidant activity. SLE had higher potential than SBE for further development as it had higher antioxidant activity and total phenolics content. Furthermore, the percentage of extraction (% yield) of SLE was higher than that of SBE, as shown in Table 1. In terms of environmental aspects, product development using leaves is favorable as they are normally discarded following harvesting of the wood for construction, furniture and agricultural use.

Moreover, SLE exhibited better melanin inhibition than SBE. The results from the anti-melanogenesis assay, which determined the anti-tyrosinase enzyme activity using L-tyrosine as a substrate, showed SLE had a half inhibitory concentration (IC_{50}) of 34.07 µg/mL which was four times better than SBE (IC_{50} = 133.55 µg / mL). However, the extracts exhibited the lower tyrosinase inhibitory activity than the standard Kojic (IC_{50} = 2.43 µg / mL) (Figure 1). In addition, neither extract, at concentration of 0.06-1.00 mg/mL, had cytotoxicity on B16-F10 melanoma cells, whereas kojic acid showed significant toxicity at its high concentration (Figure 2). Therefore, these plant extracts are safe. Moreover, at non-

toxic concentrations (25-100 µg/mL), the extracts exhibited significant melanin inhibition in B16-F10 cells. Similar to the anti-melanogenesis assay, SLE (100 µg/mL) showed significantly higher activity than SBE to inhibit melanin production ($P<0.05$) (Figure 3). The results from melanin production and cell viability tests suggest that the ability to inhibit melanin formation come from a direct effect on melanogenesis, not cell death. Furthermore, RT-PCR analyses showed the mechanism of melanin inhibition is via the down regulation of tyrosinase gene expression, TRP-1 and TRP-2. SBE and SLE at doses of 25-100 µg/mL significantly suppressed the level of these genes in B16-F10 cells at dose dependent manners with IC_{50} of 67.42-75.25, 68.67-100.84 and 88.79-91.40 µg/mL for tyrosinase, TRP-1 and TRP-2, respectively (Figure 4 and 5).

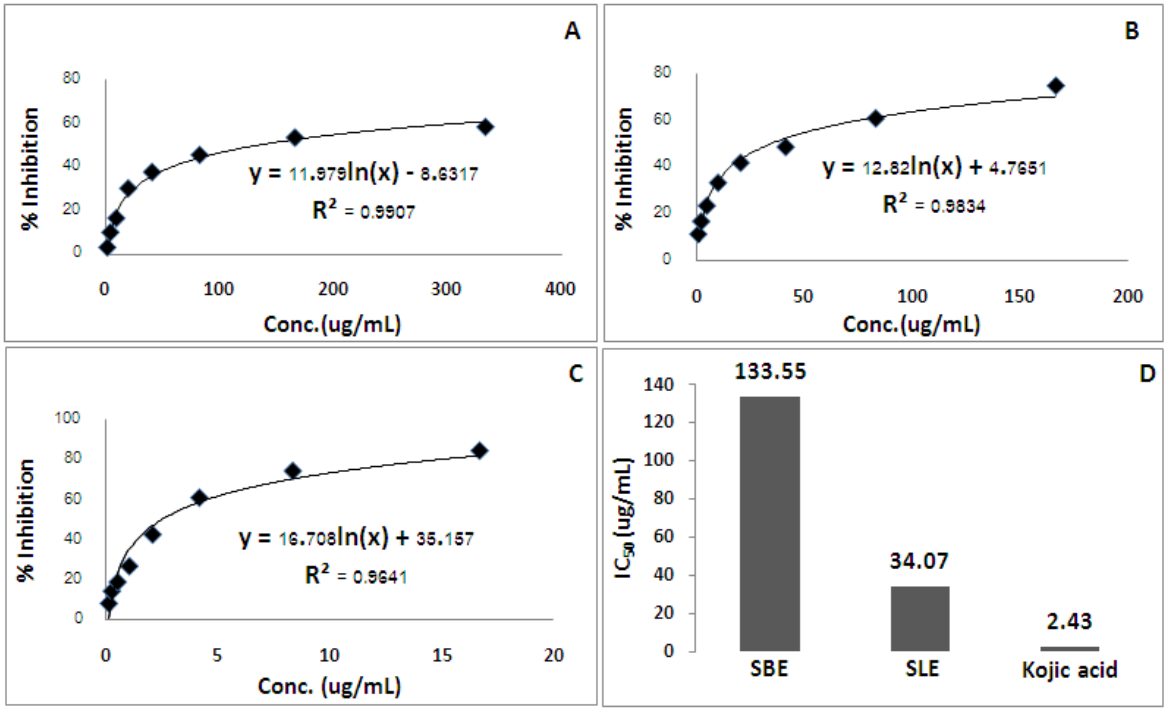

Figure 1. Effects of SBE (A), SLE (B) and kojic acid (C) on tyrosinase activity and their IC_{50} (D)

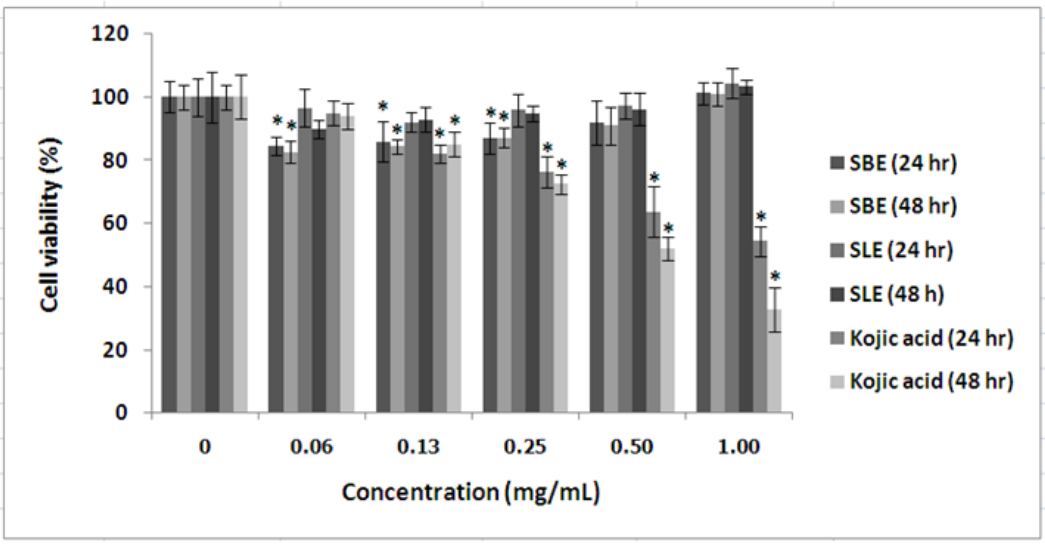

Figure 2. Effects of SBE, SLE and kojic acid on viability of B16-F10 cells. (*$P < 0.05$ vs. untreated cells)

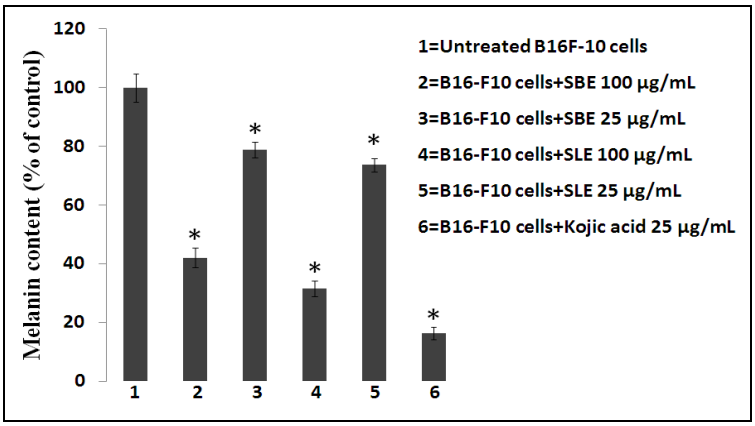

Figure 3. Effect of SBE, SLE and kojic acid on melanin production in B16-F10 cells. (*P < 0.05 vs. untreated cells)

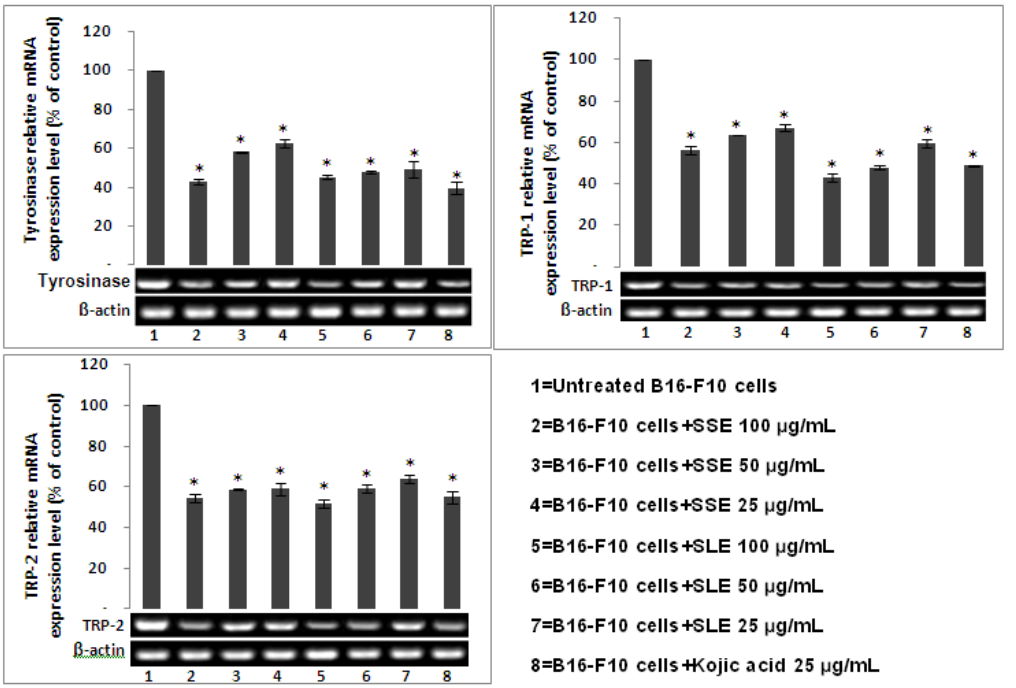

Figure 4. Effects of SBE, SLE and kojic acid on mRNA expression of tyrosinase, TRP-1 and TRP-2. (Results are expressed as % of control, *P < 0.05 vs. untreated cells)

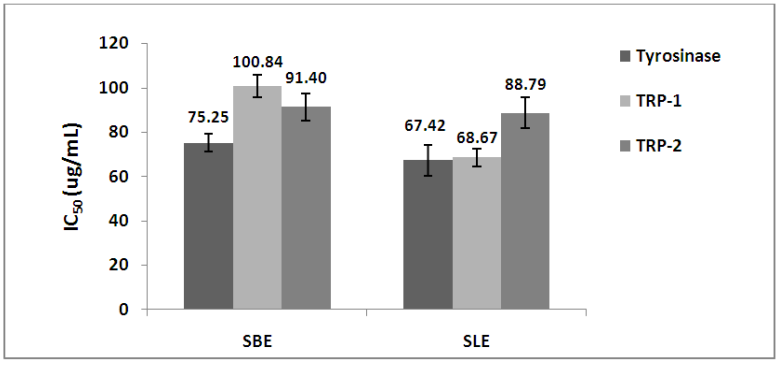

Figure 5. Half maximal inhibitory concentration (IC_{50}) of SBE and SLE on the expression of melanin production-related genes

Syzygium cumini was selected due to its ethno pharmacological effects and chemical constituents. Its chemical compounds such as quercetin, gallic acid, triterpenoids, ellagic acid and flavonol glycosides have been shown to have pharmacological benefits related to antioxidant and anti-tyrosinase activities (Chaiprasongsuk, Onkoksoong, Pluemsamran, Limsaengurai, & Panich, 2016; Eom *et al.*, 2016; Jauregui *et al.*, 2015; Rajan & Muraleedharan, 2017; Sripanidkulchai & Junlatat, 2014). The mechanisms of some chemical constituents of S. cumini such as ellagic acid on tyrosinase inhibition have been reported to be through copper chelation. Their constituents have passed clinical trial study (Fisk, Agbai, Lev-Tov, & Sivamani, 2014). Gallic acid has exhibited inhibition of both melanin synthesis and tyrosinase activity, and decreased the expression of melanogenesis-related proteins, such as microphthalmia-associated transcriptiton factor (MITF) , tyrosinase, tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase (Dct) (Su *et al.*, 2013). These compounds are normally found in plants in varying amounts depending on genetics and environment, and occur as defense mechanisms against UV-induced damage, aiding in absorbing UV radiation by accumulation of phenolic compounds in their superficial layers (Heo *et al.*, 2010). Park *et al.* (2000) suggested that due to their ability to attenuate UV damage, the phenolic compounds noncompetitively inhibited tyrosinase activity at a site other than the active site of this enzyme.

There is a close relationship between antioxidant and anti-tyrosinase activities as shown by UV light inducing oxidative stress along with increased melanin production in melanocytes (Choi *et al.*, 2005). Biochemically, this relationship depends on dopaquinone, an unstable intermediate of melanogenesis, which is converted to dopachrome by tyrosinase or autoxidation, from which melanin can be formed through a subsequent polymerization reaction. Thus, the inhibition of melanogenesis can be achieved by both antioxidation and tyrosinase inhibition. While there are many substances that inhibit tyrosinase, kojic acid was used as a positive control as it is a well-known anti-tyrosinase agent. Although kojic acid was reported to have both competitive inhibitory effect on monophenolase activity and mixed inhibitory effect on the diphenolase activity of mushroom tyrosinase (Chen, Hung, Chen, Lai, & Chan, 2016) , it also showed high cytotoxicity and chemical instability during storage which may result in skin irritation. As detected in the present study, kojic acid at concentration above 0.06 mg/mL was cytotoxic, whereas the plant extracts up to 1 mg/mL were nontoxic to the cells. Our finding implies that with nontoxicity and high antioxidation and melanin production inhibitory properties, the leaf of this plant should be further investigated for the potential use as an ingredient in skin whitening cosmetics.

4. Conclusions

In summary, the ethanolic extracts of leaf and branch of *S. cumini* exhibited anti-melanogenic effects in correlation with their antioxidant potential. This is the first report regarding this plant extracts in terms of its inhibitory effects of melanogenesis. *S. cumini* not only had antioxidant activity against oxidative stress, but also showed cosmeceutical activities, including inhibitory effects on tyrosinase activity and melanin synthesis via the suppression of tyrosinase, TRP-1 and TRP-2 genetic expressions. Hence, these results indicate that *S. cumini* could be used as an effective natural source in pharmaceutical and cosmeceutical industries.

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