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Inhibitory Effect of Ceylon Black Tea Extract on the Melanogenesis in α-MSH Stimulated B16F10 Melanoma Cells

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Abstract The desire to be light skinned is universal among women. Asia has a long history of using skincare formulations as whitening agents. There is an imperative need to develop novel cosmetics from herbal sources due to several unpleasant side effects and high costs. As a result, this study aims to investigate the effect of Ceylon black tea extracts on melanogenesis. Five different Ceylon black tea extracts were prepared and examined for total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, and tyrosinase inhibitory activity. Furthermore, B16F10 mela noma cells were treated with these extracts and tested for cytotoxicity and protein suppres sion levels. According to the results of this study, the highest TPCs were obtained from ethanol and acetone extractions (240.303 ± 1.389 μg/g and 240.202 ± 4.700 μg/g, respecti vely), whereas the highest TFC was obtained from acetone extraction (57.484 ± 0.413 μg/g). Ceylon black tea extracted with ethanol exhibited the highest inhibitory activity on tyrosinase with an IC_{50} value of 0.277 ± 0.017 mg/mL and the highest DPPH radical scavenging activity with an EC_{50} value of 0.009 ± 0.000 mg/mL. Furthermore, western blot results revealed that tyrosinase, TRP-1, TRP-2, and MITF protein expression levels were dose-dependently suppressed, indicating the applicability of Ceylon black tea extract as a novel melanogenesis inhibitor.

Keywords : Ethanol extract, Anti-melanogenic, Cosmeceuticals, Ceylon black tea, Tyrosinase

Introduction

The origin of tea dates back to ancient civilization as brews prepared by dipping tea leaves in hot water provided yummy beverage. *Camellia sinensis* belongs to family Theacea, originated in China dates back sever al thousand years as traditional medicine. Today tropica l and temperate regions of Asian, African and South American countries are the main origin of tea plant. Globally tea is the second most widely consumed bever age next to water and it is deliberated as the flavored non-alcoholic drink with therapeutic properties [1,2].

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Manufacturing process leads to some differences betwe en types of tea though they are from the same plant source. Whereas, green tea (unfermented tea), oolong tea (semi-fermented) and black tea (fully fermented) with differences in manufacturing process leads to che mical composition changes [3,4]. Globally around 78% of tea is consumed as black tea, 20% as green tea and reminder being less than 2% as oolong tea [5]. World tea production and consumption are going rising as a result of robust demand in emerging and developing countries. It had been forecasted that the global tea cons umption will reach 3.3 million tons in 2021 while more than half of tea consumption comes from Asia. Tea con sumption in Korea has been a part of daily lives and per capita tea consumption for 2018 year merely 168 grams [6]. Tea has been studied so far for its pharmacol ogical applications by preventing health consequences such as cardiovascular disease, type 2 diabetes, obesity, neurological diseases and certain type of cancers [7-9]. Therefore, in this study we will research on possible application of black tea in cosmeceuticals due to lack of information of black tea to combat skin care complic ations. Black tea contains numerous constituents such as, flavonoids, phenolic acids, amino acids, vitamins, fluoride, lipids and β-carotene. Theaflavins and thearubi gins are the major polyphenols found in black tea [2]. Scavenging ability of polyphenols has elucidated antiox idant potential of fully fermented black tea [3]. Further more, anti-collagenase activity, anti-hyaluronidase activ ity, skin whitening activity and sun screen activity of black tea extracts have investigated for their cosmeceuti cal and skin promoting activities [10-12]. The present study further investigated the inhibitory activity of Ceyl on black tea extracts on melanin synthesis at the cellular level.

Methodology

Preparation of Ceylon black tea extracts

We were mainly interested in water extraction and org anic extraction with ethanol, methanol and acetone. Firs t 4 g of Ceylon black tea was suspended in 160 mL of distilled water (Tea: Distilled water ratio w/v 1:40) and autoclaved at 121 ℃ for 15 min. Then the supernat ant was separated by centrifugation 30,000 g for 5 min followed by filtration (Hyundai, micro51, 90 mm quant itative ash less). Filtrate was lyophilized and stored in -21 ℃ until use. Another black tea sample of 25 mg/m L was extracted at 80 ℃ for 2 h. Separated supernatant from centrifugation (30,000 g for 5 min) was filtered and lyophilized to store in -21 ℃ until use. For the organic extraction 4 g of tea powder was extracted with 80% organic solvents (160 mL) for 3 days at room temperature and filtered. After filtration, the organic ext racts were evaporated under vacuum to obtain dry samp les [13].

Total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity

Then extracts were tested for total phenolic content and total flavonoid content as described by Xu *et al.*, 2009 and Bag, Grihanjali Devi and Bhaigyaba, 2015 [14, 15]. The antioxidant activity was measured by usin g the reducing power test of DPPH radicals as describe d by Blois, 1958 [16].

Tyrosinase inhibition assay

The inhibitory effect on tyrosinase was measured usin g the spectrophotometric method developed by No *et al.* (1999) [17]. A total of 10 μL of each sample solutio n with different concentrations and 20 μL of tyrosinase (110 units/ml) in 50 mM sodium phosphate buffer (pH 6.5) were added to 170 μL of an assay mixture containi ng a ratio of 10:10:9 of 1.5 mM L-DOPA solution, 50 mM sodium phosphate buffer (pH 6.5), and distilled water in a 96-well plate. After 10 min of incubation at 37 °C, the absorbance of the mixture was determined at 450 nm using a Tecan F-200 multiwell plate reader

(Tecan, Mannedorf, Zurich, Switzerland). The extent of inhibition by addition of samples was expressed as the concentration required for a 5% inhibition (IC_{50} val ue). The percentage inhibition of tyrosinase activity wa s calculated via the following equation:

$$Inhibition\ (\%) = \left\{1 - \left[\frac{(Aa - Ab)}{Ac}\right]\right\} \times 100$$

In which Aa is the absorbance at 450 nm with the test sample and enzyme, Ab is the absorbance at 490 nm with the test sample and without enzyme, and Ac is the absorbance at 450 nm with enzyme and without the test sample.

MTT cell viability assay

The B16F10 melanoma cells were acquired from Kore an Cell Line Bank (KCLB, Seoul, Korea). Cells were maintained in Dulbeco's -12–modification of eagles's medium (DMEM, Thermo Scientific Inc., Bremen, Ger many) supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum (FBS) at 37 °C in humidified incubator under 5% CO_2. Confluent cultures were wash ed 1 time with PBS and then collected with scraper. Collected cells were re-suspended in DMEM and seede d to cell culture flask or well plates.

Extract with the highest tyrosinase inhibitory activity was tested in B16F10 melanoma cells for cytotoxicity by using MTT assay as described by Hansen et al. (198 9) with slight modifications [18]. B16F10 melanoma cells were cultured in 96-well plates at a density of 1 X 10^5 cells/mL. After 24 h cells were washed with fresh medium and treated with various concentrations of samples. Then MTT solution (5 mg/mL) was added to each well and incubated for 3 h. The amount of form azan salt formed was determined by measuring the OD at 540 nm using Tecan F-200 multiwell plate reader (Tecan, Mannedorf, Zurich, Switzerland). Relative cell viability was calculated compared to the non-treatment group (OD of non-treated group − OD of treated group / OD of non-treated group × 100).

Western blot analysis

B16F10 melanoma cells were treated with different concentrations of ethanol extract of Ceylon black tea (10, 25, 50 μg/mL) and α-MSH (1 μM) for 72 h and obtained the cytoplasmic protein. Extracted proteins we re separated on 10% SDS-polyacrylamide gel electroph oresis and transferred to PVDF transfer membrane (Mil lipore, Billerica, Ma, USA). The membrane was probed with Tyrosinase, TRP-1, TRP-2, MITF and β-actin pri mary antibodies followed by secondary antibody. Finall y, blots were visualized using ChemiDoc MP imager (Bio-Rad, CA, USA) [19].

Statistical analysis

Data were analyzed in three independent experiments. Results are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was use d for comparison among means with a Tukey test follo wed by Duncan comparison. Significant difference was considered at $p < 0.05$. All statistical analyses were done with SPSS 25.0 software (SPSS Inc. Chicago, IL, USA).

Results and discussion

Extraction yield, TPC, TFC, DPPH radical scavenging activity and tyrosinasr inhibitory activity

In the present study of five Ceylon black tea extracts, highest yield of 42.93% was obtained from the ethanol extraction followed by 40.19% from acetone extraction, 39.44% from methanol extraction, 37.73% autoclave ex traction and 23.43% from water extraction at 80 ºC. The highest total phenolic contents were obtained from ethanol and acetone extractions with 240.303±1.389 μg/ g and 240.202±4.700 μg/g while the highest total flavon oid content was from acetone extraction with 57.484±0. 413 μg/g.

Ethanol extract of Ceylon black tea exhibited the high

est inhibitory activity on tyrosinase with an IC_{50} value of 0.277±0.017 mg/mL while ethanol extract exhibited the highest DPPH radical scavenging activity with an EC_{50} value of 0.009±0.000 mg/mL (Table 1). High phenolic and flavonoid contents of the ethanol extract of black tea has a significant effect on its antioxidant activity. [20, 21].

Table 1. Yield, total phenolic content, total flavonoid content, tyrosinase inhibitory activity (IC_{50}) and DPPH radical scavenging activity (EC_{50}) of Ceylon black tea extracts.

Sample	Yield (%)	TPC (μg/g)	TFC (μg/g)	EC_{50} values for DPPH radical scavenging activity (mg/mL)	IC_{50} value for tyrosinase inhibition (mg/mL)
Autoclave extract	37.733	162.323 ± 14.03^{a}	33.057 ± 14.03^{a}	0.032 ± 0.001^{a}	0.314 ± 0.028^{a}
Distilled Water extract at 80°C	23.433	154.040 ± 5.390^{a}	31.911 ± 5.390^{b}	0.033 ± 0.001^{a}	1.197 ± 0.216^{c}
Ethanol extract	42.930	240.303 ± 1.389^{c}	53.682 ± 1.389^{c}	0.009 ± 0.000^{b}	0.277 ± 0.017^{a}
Methanol extract	39.435	215.051 ± 0.975^{b}	45.297 ± 0.975^{d}	0.023 ± 0.001^{c}	0.748 ± 0.049^{b}
Acetone extract	40.193	240.202 ± 4.700^{c}	57.484 ± 0.413^{e}	0.018 ± 0.001^{d}	0.315 ± 0.013^{a}

Data express as, mean ± SD (n=3). Means within each group with different letters (a-d) differ significantly ($p < 0.05$) from each other.

Effect of ethanol extract of Ceylon black tea on cell viability of B16F10 melanoma cells

An MTT assay was used to investigate if the inhibitor would adversely induce B16F10 melanoma cell death. The amount of MTT formazan is directly proportional to the number of living cells [22]. MTT assay has been reduced by the activity of mitochondrial dehydrogenases in living cells. Consequently, the sites reduction and formation of the formazan precipitate occur where the mitochondria are located. It has been claimed that the mitochondrial succinate dehydrogenase of viable cells reduces MTT to the corresponding formazan [23]. Ethanol extracted Ceylon black tea sample used in cellular treatment at various concentrations from 10 to 50 μg/mL with 1mg/mL concentration kojic acid as the positive control. Treated concentrations were not cytotoxic to cells and exhibited more than 80% cell viability at all treated concentration (Fig. 1).

Effect of ethanol extract of Ceylon black tea on tyrosinase, MITF, TRP-1 and TRP-2 protein expression levels in B16F10 melanoma cells

Further, it was confirmed through the western blot that Ceylon black tea extract was effective against tyrosinase, TRP-1, TRP-2 and MITF. As shown in Fig. 2, protein expression of tyrosinase, TRP-1, TRP-2 and MITF decreased dose-dependently upon the Ceylon black tea extract treatment. The inhibitory effect of Ceylon black tea extract may due to the down-regulation of MITF expression. As a result, it inhibits protein expression of tyrosinase, TRP-1, TRP-2. These results suggest that ethanol extract of Ceylon black tea inhibits melanogenesis in melanoma cells. Kim et al., (2013) and Hunt et al. (1994) explained that α-MSH induces the expression of MITF, which stimulates TRP-1 and TRP-2, which increases melanin synthesis [24,25]. In the present study, western blot analysis showed that the expression of tyrosinase, TRP-1, TRP-2 and MITF was dose-dependently inhibited by treatment of ethanol extracted Ceylon black tea.

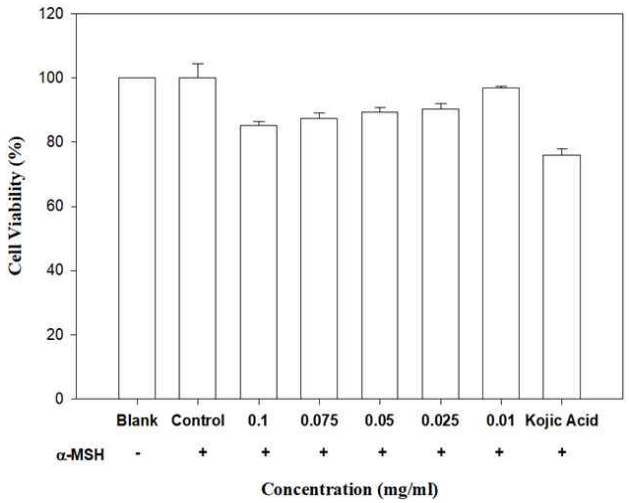

Fig. 1. Inhibitory effect of ethanol extract of Ceylon black tea on cellular melanogenesis in B16F10 melano ma cells. Cells were exposed to 1 μM α-MSH in the presence of 100, 75, 50, 25, 10 μg/mL Ceylon black tea extract or 1 mg/mL kojic acid of tyrosinase inhibito r. Each percentage value for the treated cells is reported relative to that in the control cells. Data are given as means of value ± S.D. from three independent experime nts.

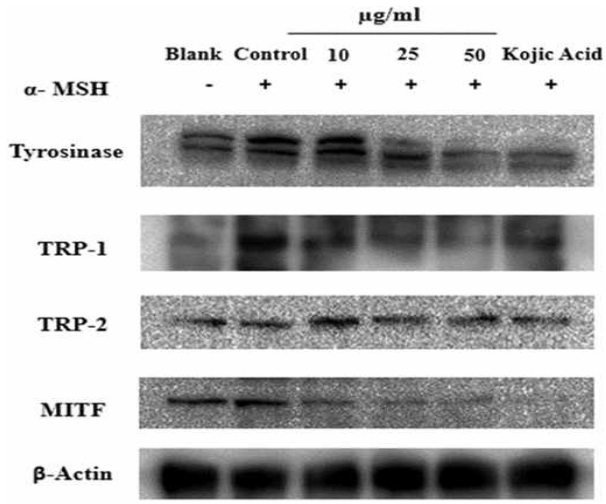

Fig. 2. Inhibitory effect of ethanol extract of Ceylon black tea on cellular melanogenesis in B16F10 melano ma cells. Cells were exposed to 1 μM α-MSH in the presence of 10, 25, 50 μg/mL concentration of extracts or 1 mg/mL kojic acid of tyrosinase inhibitor. Each perc entage value for the treated cells is reported relative to that in the control cells.

Conclusion

Numerous cosmetics in the market contain green tea infusions. However, cosmetic products containing Ceyl on black tea extracts are lacking. Cosmetic ingredients in natural products are often associated good quality, safety and prominent activity. This study demonstrated that ethanol extracted Ceylon black tea can reduce mela nogenesis in dose-dependent manner by reducing protei n levels of tyrosinase, TRP-1, TRP-2, and MITF in B16 F10 melanoma cells. Therefore, we suggest the applicab ility of the ethanol extract of Ceylon black tea as a novel inhibitor of melanogenesis with the potential to be used as a skin-whitening agent in the cosmeceutical industry.

CRediT authorship contribution statement

AUR: Writing-original draft, Methodology, Data curati on, Formal analysis. **IW:** Resource, supervision, writing -review and editing. **HGB:** Conceptualization, Formal analysis, Supervision, Funding acquisition.

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