American Journal of Biochemistry and Biotechnology

Original Research Paper

Hibiscus Flower Extract and Niacinamide Show Enhanced Whitening Effects in Mouse Melanoma Cells (B16F10)

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Article history
Received: 31-10-2022
Revised: 26-12-2022
Accepted: 07-01-2023

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Abstract: Melanoma, a type of cancer that most often occurs on skin exposed to UV rays, is becoming increasingly common. Recent research has focused actively on using natural products. The goal of this study was to compare the melanin-inhibitory effects of *Hibiscus* (*Hibiscus sabdariffa* L.) flower Extract (HE) and Niacinamide (NA), a functional ingredient with whitening effects, on melanoma cells. The 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effects of HE (1, 3.5, 7, 10, and 14 μg/mL) and NA (1, 5, 10, 20, and 25 μg/mL) on cell viability. A lactate dehydrogenase cytotoxicity assay was employed to confirm the cytotoxic effect of HE and NA on mouse melanoma cells. The real-time reverse-transcription polymerase chain reaction was performed to analyze Tyrosinase (TYR), Tyrosinase-Related Protein (TRP), and Microphthalmia-associated Transcription Factor (MITF) mRNA expression levels to determine whitening effects. Vitamin C (VC, 10 μg/mL) was selected as a control. The MTT assay showed that HE and NA were non-cytotoxic at <10 μg/mL in B16F10 cells when compared to the control, VC. The cytotoxicity of HE was confirmed to be lower than the control (VC) when a concentration of 7 μg/mL or lower was applied. The cytotoxicity of NA was low even at a concentration of 10 μg/mL. HE showed greater whitening effects at lower concentrations (i.e., 1 μg/mL) than VC (10 μg/mL). HE had almost no cytotoxicity compared to NA and VC, and the expression levels of whitening inhibition genes MITF and TYR were higher than VC, indicating that it can be used as a healthy functional food or cosmetic material.

Keywords: Melanoma, Whitening, *Hibiscus* Flower Extract, Niacinamide, B16F10, Melanin, Functional Ingredient

Introduction

Melanoma, a type of cancer, originates from melanocytes, which are present in the skin and mucous membranes of the body (Oh *et al*., 2018). Therefore, melanoma can also occur in body areas other than the skin, but most often occurs on skin exposed to ultraviolet rays. Currently, the incidence and mortality rates of melanoma are steadily increasing with an increase in human life expectancy and in the elderly population with accumulated skin damage (Lens and Dawes, 2004; Rigel *et al*., 2010). Although melanoma comprises only approximately 4% of all skin cancers, it accounts for more than 80% of deaths due to skin cancer. Furthermore, the 10-year survival rate for metastatic melanoma is less than 10% (Bhatia *et al*., 2009). Pigmentation is a key symptom of skin cancers such as melanoma and has been the subject of many studies from medical and cosmetic points of view.

Hibiscus sabdariffa L. is relatively easy to grow compared to other plants and can be used for food and fiber, making it an ideal plant to grow in developing countries. In China, the seeds are used for oil, and the plant is used for medicinal purposes and tea. In West Africa, the leaves and powdered seeds are used in cooking. It is also used in the pharmaceutical and food industries (Da-Costa-Rocha *et al*., 2014). The genus *hibiscus* belongs to the family *Malvaceae* and the plant is native to tropical Asia and West Africa (Cho and Lee, 2015). *Hibiscus* flower Extract (HE) is usually obtained by using water (Cho and Lee, 2015). HE contains substances such as malic acid and citric acid (Cho, 2015). In addition, the *hibiscus* flower is rich in Vitamin C (VC)

and contains several minerals such as potassium, calcium, iron, and magnesium; it also contains gossypetin, a type of flavonoid, as a functional ingredient (Cho, 2008). *Hibiscus* has a high potential for use as a physiologically active material because it aids in processes such as recovery from eye fatigue, strengthening of digestive function, tonicity, diuresis, bleeding prevention, and alleviation of urinary system inflammation (Choi, 2008). Another paper by Kang *et al.* (2007) confirmed the antibacterial effect of *hibiscus* ethanol extract on skin pathogens. Functional cosmetics aid in wrinkle improvement and whitening and have anti-aging effects. The functional ingredients added to such cosmetics are extremely diverse (e.g., Niacinamide [NA]) and are attracting the attention of researchers as they have both antioxidant and whitening effects (Kim and Lee, 2014).

In light of the growing demand for materials with superior functional ingredients that possess anti-aging and anti-wrinkle properties, research efforts have been directed toward the development of novel cosmetic materials. However, because of emerging safety issues with numerous materials used in functional cosmetics, the use of such materials has declined; only limited functional raw materials satisfy the effectiveness and safety criteria (Cho *et al*., 2019). Therefore, to develop novel functional cosmetic materials, research is being conducted on identifying plants and physiologically active substances derived from natural materials (Kim, 2004). The current study aims to evaluate the melanin inhibitory effect of HE, NA, and VC on melanoma cells. These functional ingredients are shown to have whitening effects and were selected for comparison in this study.

Materials and Methods

Materials

Hibiscus sabdariffa flower extract (*H. sabdariffa* L. flower, cultivated in Nigeria) and NA were provided by Innotech (Daejeon, Republic of Korea). Fetal Bovine Serum (FBS) and penicillin were purchased from Gibco (NY, USA). Mouse melanoma cells (B16F10) were purchased from the Korea cell line bank (Seoul national university, Seoul, republic of Korea). Dulbecco′s Modified Eagle′s Medium (DMEM; Gibco). To analyze the effect of HE and NA on cell viability, a 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay was performed using an MTT assay kit (Cell Proliferation) (Abcam, Boston, USA). Lactate Dehydrogenase (LDH) analysis was performed using a Quanti-LDH™ PLUS Cytotoxicity Assay Kit (Biomax, Seoul, Republic of Korea).

Methods

Cell Culture

B16F10 cells (Seoul National University, Seoul, Republic of Korea) were maintained in DMEM supplemented with a mixture of 10% heat-inactivated FBS and 1% penicillin-streptomycin at 36°C in a humidified atmosphere of 5% CO_2 and cultured for less than 15 passages.

MTT Assay for Cell Viability

B16F10 cells were seeded in 96-well plates (SPL, Seoul, Republic of Korea) at a density of 1×10^4 cells/well and then incubated at 36°C for 18 h. Each sample was diluted in DMEM to obtain final concentrations of 1, 3.5, 7, 10, and 14 μg/mL for HE and 1, 5, 10, 20, and 25 μg/mL for NA and incubated at 36°C for 24 h. Each sample was diluted in DMEM to maintain favorable conditions for cells. After incubation, MTT and serum-free Medium (DMEM) were added to each well in a 1:1 ratio and the plate was further incubated at 36°C for 3 h. The precipitate was dissolved in MTT solvent and absorbance was measured at 590 nm using a flex station 3 multi-mode microplate reader (Molecular Devices, California, USA).

LDH Cytotoxicity Assay

Cells were seeded as mentioned in the previous section. The same concentrations of HE and NA were used. Each sample was diluted in DMEM to not affect the cells. After incubation, 100 μL of each concentration was dispensed into different plates. Next, 10 μL lysis solution was added per well to the negative control in the culture plate, and 100 μL LDH reaction mixture was added to each well and then mixed. Subsequently, the plate was incubated for 30 min at room temperature (15-25°C) with protection from light. Subsequently, 10 μL stop solution was added to each well and gently mixed, following which absorbance was measured at 490 nm using a flex station 3 multi-mode microplate reader (molecular devices).

Calculation

Cell cytotoxicity (%) = (Absorbance of sample addition group-Absorbance of background control)/Absorbance of control group absorbance of background control) × 100 cDNA Synthesis and real-time Reverse Transcription (RT) Polymerase Chain Reaction (PCR).

For RNA extraction, B16F10 cells were seeded at 1×10^4 cells/well in 96 well plates (SPL) for 18 h at 37°C. Next, HE (1, 3.5, 7, 10, and 14 μg/mL) or NA (1, 5, 10, 20, and 25 μg/mL) at the final concentration was added to the wells. Total RNA was extracted using TRIzol® reagent (Ambion, USA), as per the manufacturer's instructions. The chloroform and 2-propanol used during this process were obtained from Sigma-Aldrich (St Louis, MO, USA) and Thermo Scientific (Waltham, MA, USA), respectively. The RNA obtained (10 μg) was reverse transcribed to cDNA using the revert aid first strand cDNA Synthesis Kit (Thermo Scientific) as per the manufacturer's instructions.

Table 1: Genes and primer sequences used in the present study

Gene	Primer sequence	Function
TYR: Tyrosinase	5′-AGCCATCTTGTGGGTTCTGG-3′5′-GCTTCTACCCTTGCGTGACT-3′	Melanin synthase
TRP: Tyrosinase Related Protein	5′-ATGTGCTGGAACCACGTTCT-3′5′-ATCTCAGGACTGTGTGGGACT-3′	Melanin synthase
MITF: Microphthalmia Associated Factor	5′-GAGCGTTACCAACGGAACAG-3′5′-GGGGAGAGGCACTTTACAGG-3′	Microphthalmia associated transcription factor
GAPDH	5′-TGTGACAGTGACTTGGGACAA-3′5′-CTCCTTGGAGGCCATGTAGG-3′	Housekeeping gene

Real-time PCR was performed in a 96-well plate in a CFX Opus 96 real-time PCR system (Bio-Rad, California, USA). The primers were synthesized by Oligo Synthesis (Bioneer, Daejeon, Republic of Korea). The four whitening genes that we wanted to identify include Tyrosinase (TYR), Tyrosinase Related Protein (TRP), Microphthalmia-associated Transcription Factor (MITF), and *GAPDH*. Primer sequences have been listed in Table 1. The reaction mixture (50 µL) was as follows: 2X SYBR® Green PCR Master Mix Thermo Scientific (Waltham, MA, USA), forward primer, reverse primer, cDNA, and distilled water. qPCR was performed for relative quantitation. The PCR conditions were as follows: 95°C for 10 min, followed by 39 cycles each of 95°C for 15 sec and 60°C for 1 min.

Statistical Analysis

All the data represent the mean of a minimum of three replicates and are presented as mean ± Standard Error of the Mean (SEM). Statistical analysis was performed using unpaired one-way Analysis of Variance (ANOVA) along with the Student's t-test and Bonferroni correction, using Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA). Statistical significance was set at $p<0.05$.

Results

MTT Results for HE and NA

To confirm the cytotoxicity of the HE and NA, an MTT assay was performed to check B16F10 cell viability (Fig. 1). The results showed that both HE and NA were not cytotoxic when used at <10 µg/mL concentrations. Cell proliferation was confirmed at 7 and 10 µg/mL concentrations of HE and NA, respectively. Cell proliferation was also observed at high concentrations of 10 and 20 µg/mL for HE and NA, respectively (Fig. 1).

The MTT cell viability effects of HE and NA on B16F10 are illustrated in a bar graph. As a control, only normal cells were cultured and VC was treated with 10 µg/mL. The results of three repeated experiments are shown with error bars.

LDH Results for HE and NA

An LDH cytotoxicity assay was performed to confirm the toxic effects of HE and NA on B16F10 cells. Absorbance was measured at 490 nm. The cytotoxicity of HE was lower than that of the control at a concentration of 7 µg/mL or lower. The cytotoxicity of NA was low at 10 µg/mL (Fig. 2). The results of three repeated experiments are shown with error bars.

The LDH cell cytotoxicity effect of HE and NA of B16F10 are shown in a bar graph. As a control, only normal cells were cultured and VC was used at 10 µg/mL. The results of three repeated experiments are shown as error bars.

RT-qPCR

The RT-PCR Cycle Threshold (CT) values for each sample concentration are listed in Table 2. Larger CT values indicate a whitening effect. HE showed a marked whitening effect at a lower concentration (i.e., 1 µg/mL) than that of VC (the control group), which was used at 10 µg/mL (Fig. 3). When NA was used at 1 µg/mL concentration, it had almost no whitening effect when compared with that of VC. At a concentration of 10 µg/mL, the whitening effects of HE and NA were not as marked as those of VC; however, their whitening effects were greater than those of the general control.

The results of the CT values obtained from gene expression when HE, NA, and VC were used at a concentration of 1 µg/mL are illustrated in Fig. 3. At a concentration of 1 µg/mL, HE demonstrated a higher CT value than VC and NA, which are well-known for their whitening properties. Therefore, at low concentrations, HE is considered a material that can affect whitening. HE was confirmed to have a greater whitening effect than VC when used at a concentration of 1 µg/mL, whereas NA had almost no whitening effect at this concentration.

Table 2: RT-PCR CT values for each sample concentration

Sample/genes (µg/mL)	TRP	Tyrosinase	MITF	GAPDH
HE 1	28.7	28.5	30.7	29.7
HE 3.5	26.2	24.9	27.0	26.9
HE 7	27.7	26.7	28.4	27.9
HE 10	27.3	26.4	29.0	28.3
HE 14	25.9	25.5	27.3	25.9
NA 1	25.9	23.1	25.6	24.6
NA 2	26.3	24.4	27.0	26.7
NA 5	25.1	23.2	25.8	25.1
NA 10	26.9	24.8	27.8	28.9
NA 25	26.5	25.4	27.8	26.8
Control	26.2	24.9	27.2	25.9
VC 10	27.9	27.9	30.4	28.1

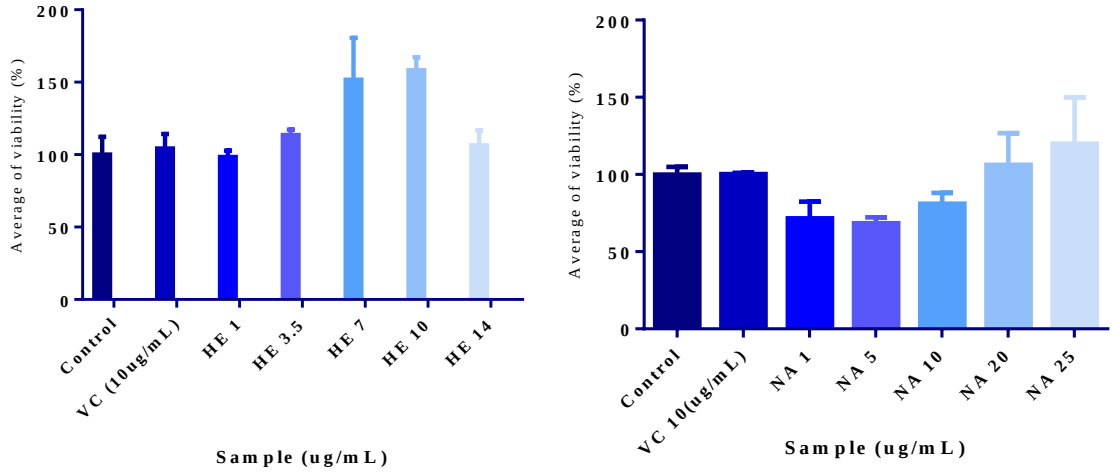

Fig. 1: MTT assay results for *Hibiscus* flower Extract (HE) and Niacinamide (NA)

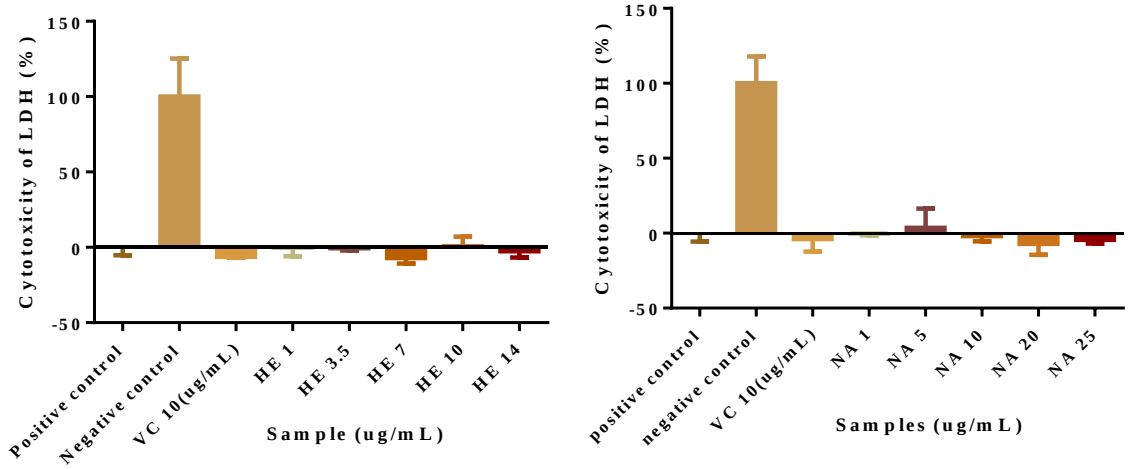

Fig. 2: LDH cytotoxicity results for *Hibiscus* flower Extract (HE) and Niacinamide (NA)

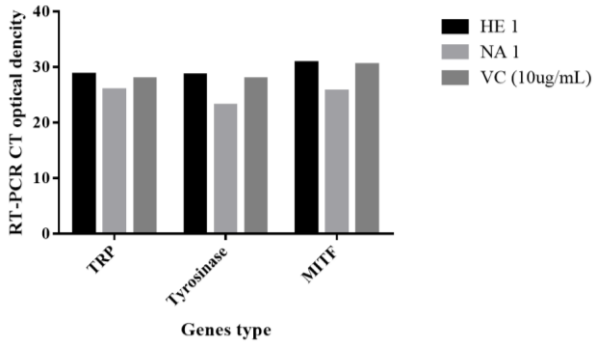

Fig. 3: RT-PCR Cycle Threshold (CT) values of *Hibiscus* flower Extract (HE) and Niacinamide (NA)

Discussion

The results of the present study confirmed that HE slowed down the expression of genes affecting pigmentation even at low concentrations when compared with NA, a whitening functional ingredient, and VC, which is well known for being effective for whitening. The MTT colorimetric method is useful for evaluating cell proliferation, cytotoxicity, and cellular metabolic activity. The MTT assay is based on the reduction of a yellow tetrazolium salt to water-insoluble purple formazan by cellular redox enzymes (Gerlier and Thomasset, 1986; Mosmann, 1983). Therefore, the MTT assay is based on enzymatic activity in cells (Gerlier and Thomasset, 1986). However, it can be considerably influenced by certain confounding factors, including cell culture (Benov, 2019). To confirm the cytotoxicity of HE and NA, the survival rate of B16F10 cells was measured after performing the MTT assay. Cell proliferation was confirmed when 7 and 10 µg/mL of HE was used. The cytotoxicity of NA was weak even at 20 µg/mL. Findings from the comparison between NA and HE confirmed that HE has the potential used as a functional cosmetic material at low concentrations. In a study conducted with a solvent extracted from the dried leaves of *hibiscus*, it was demonstrated that the main component of HLP is ECG and that polyphenols including ECG have antioxidant, anti-inflammatory, and anticancer properties (Chiu *et al*., 2015). Confirming the expression of genes involved in whitening in melanoma cells, we found that HE was more effective at suppressing melanin than NA or VC at low concentrations. In previous studies, leaves of *H. cannabinus* L. were used to prepare a cosmetic formulation for melanin suppression (Sim *et al*., 2022). It was effective in the expression of TYR, TYP-1, TYP-2, and MITF.

Conclusion

The HE showed little toxicity compared to NA and VC and the expression level of whitening inhibition was higher than that noted with VC. Therefore, the findings indicate that it can be used as a cosmetic material or healthy functional food. In addition, since there are few studies in which HE has been applied to the skin, our findings provide basic data that could facilitate the mixing of HE with other extracts and complexes. Nevertheless, additional studies are required to measure mushroom tyrosinase activity or melanin expression following the application of HE to the skin, in addition to gene expression tests associated with whitening.

Acknowledgment

Thank you to the publisher for their support in the publication of this research article. We are grateful for the resources and platform provided by the publisher, which have enabled us to share our findings with a wider audience. We appreciate the efforts of the editorial team in reviewing and editing our work, and we are thankful for the opportunity to contribute to the field of research through this publication.

Funding Information

This research did not any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author's Contributions

Jae Eun Choi, Tae Su Jang and Jae Kyung Kim: Made substantial contributions to the conception and designed of the study.

Jung Min Park and Eun Ji Park: Made substantial contributions to the acquisition and analysis of the data.

Ethics

This study was approved by the Dankook University Institutional Review Board (IRB file No. 2022-002-002).

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