INHIBITOR EFFECT OF FLAVONOID FROM *BLUMEA BALSAMIFERA* (L.) DC. LEAVES EXTRACT ON MELANIN SYNTHESIS IN CULTURED B16F10 CELL LINE AND ZEBRAFISH

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ASTRACT

Tyrosinase is involved in melanin biosynthesis and the abnormal accumulation of melanin pigments leading to hyperpigmentation disorders that can be treated with depigmenting agents. EA fration isolated from *Blumea balsamifera* (L.) DC leaves demonstrate that 200 mM of EA apparently attenuates 41.8% melanin content of human normal melanocytes without significant cell toxicity. Moreover, the zebrafish *in vivo* assay reveals that EA effectively reduces melanogenesis with no adverse side effects. These results evident that EA isolated from *Blumea balsamifera* (L.) DC leaves is a promising candidate in developing pharmacological and cosmetic agents of great potency in skin-whitening.

Keywords: *Blumea balsamifera,* ethyl acetate fration, B16F10 melanoma, skin-whitening.

INTRODUCTION

Melanin overproduction and accumulation had been reported to be the causes for cutaneous hyperpigmentation in mammals (Slominski et al., 2004). Melanin biosynthesis is initiated with tyrosine oxidation by tyrosinase, the enzyme which catalyzes the rate-limiting step for melanogenesis (Hu et al., 2015). Upon exposure to ultraviolet radiation, melanin formation plays a critical role in protecting skin from UV-induced DNA damage (Brenner and Hearing, 2008); however, abnormal melanism such as freckles, solar lentigines and dark spots are regarded as aesthetically unfavorable (Yoon et al., 2009), having been significantly promoting the development of cosmetic products. Therefore, tyrosinase inhibitors have received great attention in the field of cosmetics due to the amelioration of skin pigmentation disorders. To date, several skin depigmenting agents, such as kojic acid and arbutin, are being sold as commercially available products. Nevertheless, due to the concerns related to adverse effects and long-term effectiveness, novel tyrosinase inhibitors with higher activity, lower toxicity and sufficient penetrative ability are still needed (Choi et al., 2007).

Blumea balsamifera is a medicinal plant that grows in Southeast Asia, belongs to the family Asteraceae. The leaves are also used as a tea, and as a cure for certain disorders such as rheumatism and hypertension. Its leaves have attracted attention as a part of the plant with various physiological activities, including plasmin-inhibitory, antifungal, and liver-protective effects (Norikura et al., 2008), antidiabetic (Roy et al., 2013), wound healing (Krishna et al., 2015), angiogenic (Mistry et al., 2015). Moreover the methanol extracts of the leaves of this plant exhibited antibacterial (Nessa *et al*., 2004b), free radical scavenging activities (Nessa *et al*., 2004a), lipid peroxidation inhibitory activities (Nessa *et al*., 2003), xanthine oxidase inhibitory, superoxide scavenging activities (Nessa *et al*., 2010) and antityrosinase (Saewan et ai., 2011; Thach et al., 2017). These activities were attributed due to the presence of

several flavonoids in the leaves extract of this plant. Phytochemical investigation on the leaves of *B. balsamifera* resulted in the isolation and characterization of 11 flavonoids viz. velutin, dihydroquercetin-7,4′- dimethyl ether, blumeatin, ombuine, tamarixetin, rhamnetin, luteolin-7-methyl ether, luteolin, quercetin, 5,7,3′,5′-tetrahydroxyflavanone and dihydroquercetin-4′- methyl ether (Lin *et al*., 1988; Nessa *et al*., 2004a). Inside, dihydroquercetin-7,4′-dimethyl ether (I), dihydroquercetin -4′-methyl ether (II), 5,7,3′,5′-tetrahydr oxyflavanone (III), blumeatin (IV) and quercetin (V) were five major flavonoids of *B. balsamifera* (fazilatun et al., 2013). In this study, we investigated the inhibitory effect of flavonoid from *Blumea balsamifera* (L.) DC leaves extract on melanin synthesis through identify melanogenic inhibitory effect in B16F10 cell line and zebrafish embryo.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained as pure commercial products and used without further purification. Hexan, ethanol, ethyl acetate were from Duchefa; arbutin, kojic acid were from Sigma Chemical Co (St. Louis, USA).

Preparation of Extract

B. balsamifera DC leaves were only used that has grown in the medicinal garden of Institute of Tropical Biology- Vietnamese Academy Science and Technology. Leaves was washed, dried at 50^{o}C and grinded to dust. 200 g of powder was soaked in 600 mL of ethanolic at temperature 80^{o}C for 90 minutes. The extract was evaporated to dryness under reduced pressure to give the ethanolic extract. Ethanolic extract (5 g) was suspended in water (100 ml) and re-extracted with hexane (100 ml) , and ethylacetate (100 ml) respectively. Each soluble fraction was evaporated under reduced pressure to give the hexane (1.1 g), ethylacetate (2.35 g), and water (1.55 g) extracts. Each extract was dissolved in DMSO and idenfied flavonoid content following the method of Quettier et al., (2000). The fraction ethyl acetate (FE) was used for the further biological study including melanogenic inhibitory effect in B16F10 cell line and zebrafish embryo.

Cell viability and Melanin quantification assay

B16F10 melanoma (ATCC-CRL6475) cells were cultured in DMEM supplemented fetal bovine serum (FBS) 10% (v/v) and penicillin/ streptomycine 1% (v/v). Then the cells were placed in 96-well plates (6.10^{4} cells/well) for cell viability assay and 24-well plates (13.10^{5}/well) for melanin content assay. After 24 hours, the cells were respectively treated with arbutin (200 mM) and EA (12,5; 25; 50; 100 and 200 mM) at 37^{0}C and incubated for 72 hours. From measurement of melanin contents, 1N hot NaOH (70^{0}C) was used to and dissolved the melanocyte pellets for 1 hour and further centrifuged at 10,000 x g for 10 min. The microplate reader (Molecular Devices Spectra Max M2) was employed to determine the optical density (OD_{405}) of each supernatant. Cell viabilities were determined by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) method (Jone et al., 2002).

Zebrafish *in vivo* assay

Zebrafish *in vivo* assay was performed according to the previous method (Choi et al., 2007). The collected synchronized zebrafish embryos were arrayed by pipette into a 96-well plate, three embryos per well with 200 ml embryo medium. The prepared inhibitor solutions (in 1% DMSO) were added to the embryo medium from 9 to 57 hpf (hours post fertilization, total 48 h exposure). The positive controls were 25 µM kojic acid. Stereomicroscope was employed for observing the effects on the pigmentation of zebrafish. Phenotypebased evaluation of the body pigmentation was performed at 57 hpf. Embryos were deteriorated by forceps,

anesthetized in tricaine methanesulfonate solution (SigmaAldrich), mounted in 1% methyl cellulose on a depression slide (Aquatic EcoSystems, Apopka, FL, USA) and photographed under the stereomicroscope Z16 (Leica Microsystems, Ernst-Leitz-Strasse, Germany) for observation. Images were captured using a SPOT CCD Idea integrating camera (Diagnostic Instruments Inc.: Sterling Heights, MI, USA). Afterwards, images were taken using the ImageJ software (Version 1.40 g, National Institutes of Health, Bethesda, MD) by a blinded observer. The pixel measurement analyzer program was then used to count the area of the zebrafish image pigmentation. The quantification of pigmentation data was expressed as a percent change compared to the control group, which was considered 100%. (Eric et al., 2010).

RESULTS AND DISCUSSION

Flavonoid content

The concentration of flavonoids in leaf extract of *B. balsamifera* DC.was determined using spectrophotometric method with aluminum chloride. The flavonoid content in fraction ethyl acetate extract (EA) is the highest among those extracts (Table 1). Saewan et al (2011) showed that The ethylacetate extract showed the highest tyrosinase inhibitory activity among hexan and water extracts. So that, the ethyl acetate extract was used to melanogenic inhibitory effect in B16F10 cell line and zebrafish embryo.

Table 1. Flavonoid content in fractions extract from *B. balsamifera* DC.

Fraction extract	flavonoid content (mg/g)
Hexan	52.88
Ethyl acetate	130.10
H_2O	38.29

Cell viability and Melanin quantification assay

The inhibitory effect of EA on human melanin formation was investigated. The change of melanin content in the normal human melanocyte cells treated with 12,5; 25; 50; 100 and 200 mM of EA was examined individually to determine the depigmentation activity. The result shows that 100 and 200 mM of EA sufficiently attenuate (30,8% and 41,8 %) melanin content (Fig. 1a, b), whereas there is little decrease (20%) of the melanin content at the presence of 200 mM of arbutin. Moreover, to evaluate the cytotoxic effect of EA, MTT assay was applied to investigating whether the EA would induce cell death adversely. The effect of EA on cell viability was shown in Fig. 2. At a dose of 100 and 200 mM of EA, cell viability is 99.2% and 97.2% respectively, almost identical to those of arbutin. Thus, it is believed that EA is non-cytotoxic to normal human melanocyte cells in the concentration ranging from 12,5; 25; 50; 100 and 200 mM.

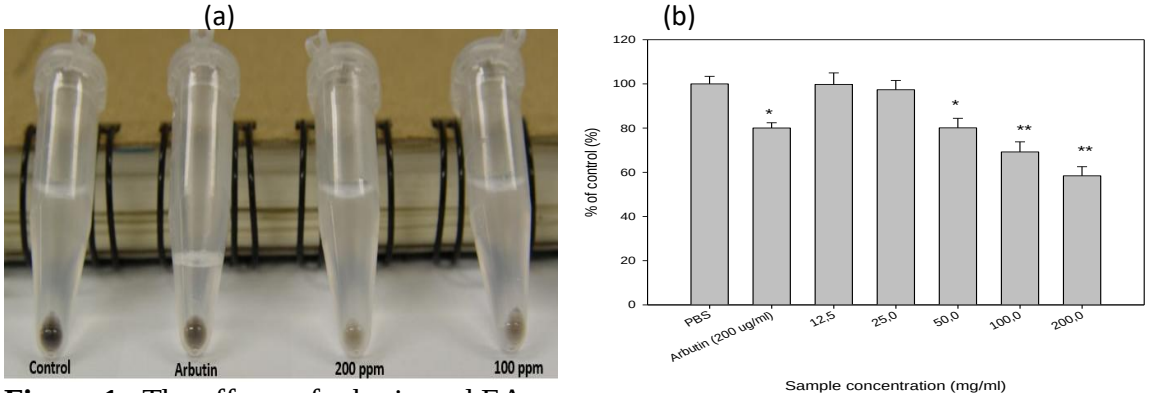

Figure 1 . The effects of arbutin and EA on melanogenesis of human melanocyte: (The melanin contents of arbutin, and EA treated human melanocytes with 12,5; 25; 50; 100 and 200 mM. DMSO indicates buffer control.

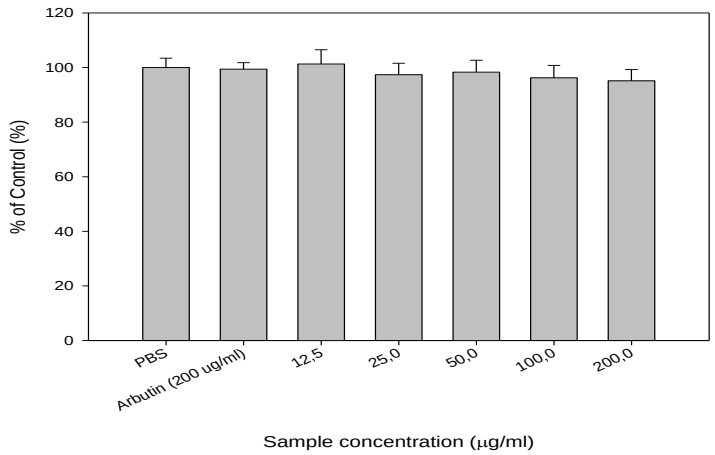

Figure 2. The effects of arbutin, and EA on cell viability of human melanocyte.

Zebrafish (*in vivo* assay)

In addition to estimating the effects of EA on cell viability and human melanogenesis, we further evaluate its anti-pigmentation ability through *in vivo*. Zebrafish is an particularly useful vertebrate model organism because it possesses similar gene sequences and organ systems to human beings (Veldman and Lin. 2008). With these advantages, the zebrafish system was employed to investigate the *in vivo* melanogenic inhibition (Choi et al., 2007), and the EA toxicity was also determined simultaneously. The inhibition effects of EA on the pigmentation of zebrafish were evaluated. Kojic acid were used as control groups. With the treatment of 10, 100, 200, 300 mM EA, the pigmentation level of zebrafish obviously decreases about 13.6; 40.2; 49.3 and 74.0% (Fig. 3a,b). However, the de-pigmenting effects (55.5%) at the presence 25 μM of kojic acid is individually much lower than that of 300 mM EA, as shown in Fig. 3b.

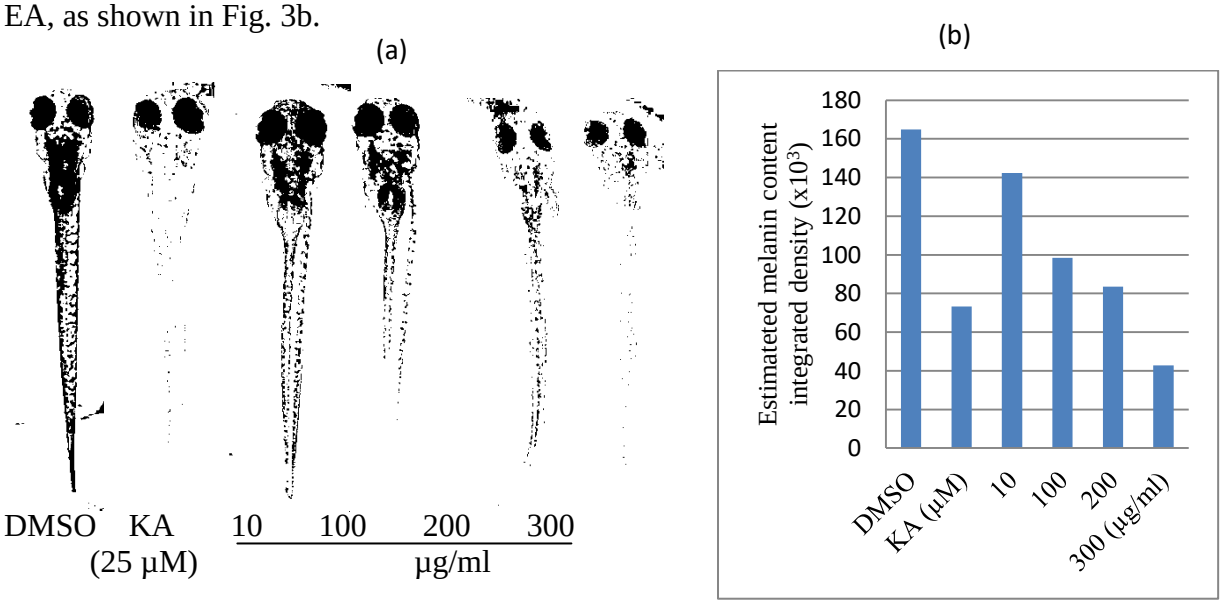

Figure 3. Estimation and comparison of depigmenting effects of kojic acid and EA by *in vivo* zebrafish assay. (a) Illustration of the pigmentation levels of zebrafish treated with EA (10, 100, 200,300 mM) and kojic acid (25 μM). (b) Comparison of the depigmenting effects of kojic acid and EA at 10, 100, 200, and 300 mM. KA represents Kojic acid.

CONCLUSION

This study identified the potent for melanogenic inhibition, and depigmentation. The *in vitro* assay demonstrates that 200 mM of EA apparently attenuates 41.8% melanin content of human normal melanocytes and shows no significant cell toxicity. The *in vivo* assay reveals that EA effectively reduces melanogenesis in zebrafish without any adverse side effects. In conclusion, EA isolated from *Blumea balsamifera* is a potential candidate in developing safe cosmetic and pharmacological agents, which is of great potency in skin-whitening.

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