Comparative Evaluation of Antityrosinase and Antioxidant Activities of Dietary Phenolics and their Activities in Melanoma Cells Exposed to UVA

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ABSTRACT

Background: Dietary phenolics have been shown to possess antityrosinase and antioxidant properties which account for their pharmacological effect against ultraviolet (UV)-mediated skin pigmentation. Hence, this study assessed the correlation between antityrosinase and antioxidant activities of various phenolic acids including caffeic acid (CA), ferulic acid (FA), gallic acid (GA), *p*-coumaric acid (PA) and quercetin using cell-free systems including mushroom tyrosinase and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays and human melanoma (G361) cell culture model.

Methods: Antityrosinase and free radical (FR) scavenging activities of all test phenolics were determined using mushroom tyrosinase and DPPH assays, respectively. Inhibition of cellular melanogenesis with regard to regulation of intracellular oxidant formation and glutathione (GSH) content was assessed in UVA-irradiated G361 melanoma cells.

Results: The IC_{30} values for the mushroom tyrosinase inhibition activity showed a rank order of quercetin ≈ PA > kojic acid (KA) ≈ CA ≈ FA > GA. For the FR scavenging activity, IC_{30} values demonstrated a rank order of GA ≈ CA ≈ FA > quercetin > PA ≈ KA. In addition, both CA and FA were observed to suppress UVA-induced tyrosinase activity and melanin content in G361 cells, although CA exerted greater antimelanogenic effect than FA. Pretreatment with CA was also able to reduce oxidant generation and restore GSH content in irradiated cells.

Conclusion: Cell-free systems showed that antityrosinase activity of test phenolics was not associated with their FR scavenging activity. Moreover, we have herein reported the correlation between depigmenting effect and antioxidant action of CA in G361 cells.

Keywords: Phenolic acids, antioxidant, tyrosinase, melanogenesis, glutathione

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INTRODUCTION

Dietary phenolics have been attractive for dermatology research since they possess powerful antioxidant properties, which might be responsible for their inhibitory effect on skin hyperpigmentation. Melanin plays an essential role in protection against UV irradiation-induced skin damage, although abnormal accumulation of melanin can result in dermatologic problems including malignant melanoma and cosmetic concern. Recently, an underlying mechanism of melanogenesis involved in oxidative stress has been proposed[1] and attempts have thus been made to investigate the antimelanogenic effect of natural products-derived antioxidant properties. Tyrosinase is a copper-containing monooxygenase which accounts for melanin formation in melanocytes and/or melanoma cells and so has become a key target for screening of novel whitening agents. Cell-free system assays of antityrosinase and FR scavenging activities are spectrophotometric methods which have been widely employed to screen promising whitening and antioxidant agents, respectively, because such techniques are sensitive, rapid, convenient and inexpensive.[2,3]

UVA has been postulated to contribute to skin pigmentation through oxidative stress, which takes place when there is an increase in cellular oxidant production.[4]

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Moreover, previous reports have suggested that compounds having abilities to inhibit oxidative stress would be useful in regulation of melanogenesis through mitigating tyrosinase activity and melanin synthesis.[5,6] Since antioxidant action of putative whitening agents may be involved in their antityrosinase effects, the objective of our study was therefore to investigate the correlation between antityrosinase and antioxidant activities of various phenolic acids including caffeic acid (CA), ferulic acid (FA), gallic acid (GA), *p*-coumaric acid (PA) and quercetin as well as kojic acid (KA), a well-known tyrosinase inhibitor (Fig 1). Different classes of phenolics were tested in this study. CA, FA and PA are cinnamic acid derivatives, GA is a benzoic acid derivative and quercetin is a flavonoid. Cell-free systems including mushroom tyrosinase and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays and human melanoma (G361) cells irradiated with UVA were used to evaluate antityrosinase and antioxidant actions of test phenolics.

MATERIALS AND METHODS

Materials

Chemicals and reagents of the highest quality available were used and obtained from Sigma-Aldrich (MO, USA or Germany). Human melanoma cell lines (G361) from American Type Culture Collection (ATCC, Rockville, MD, USA) was a kind gift from Assoc. Prof. Tengamnuay, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Cell culture medium and reagents were purchased from Invitrogen (NY, USA).

Mushroom tyrosinase assay

The activity of mushroom tyrosinase was assayed as previously described[1] using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate and KA was used as a reference compound. The sample solution (20 μl) and mushroom tyrosinase (40 μl) in 20 mM phosphate buffer (480 units/ml) were added to PBS (120 μl, 20 mM) in a 96-well plate. The reactions were initiated by adding L-DOPA (20 μl) and the reaction mixture was further incubated for 5 min at 25°C. Then, the optical density (OD) of the reaction mixture, which was proportional to the amount of dopachrome produced, was determined at 470 nm. The percentage inhibition of tyrosinase activity was calculated using the following equation; [1-(C-D)/(A-B)] x 100, in which A represents the OD of the reaction mixture containing the enzyme without the test sample, B represents the OD of PBS only, C represents the OD of the reaction mixture containing the enzyme and the test sample and D represents the OD of the reaction mixture containing the test sample without the enzyme.

Measurement of free radical scavenging activity

DPPH is a stable free radical commonly used to determine FR scavenging activity.[13] Briefly, test samples (100 μL) in 80% ethanol were added to a solution of 0.2% (w/v) DPPH radical (100 μL) in ethanol in a 96-well plate. The absorbance was monitored spectrophotometrically at 520 nm at 0 and 15 mins by a microplate reader. The FR scavenging activity was evaluated as a decrease in the absorbance of DPPH radical and the scavenging activity percentage was calculated using the equation; $[(A_0-A_{15})/Ac] \times 100$, in which A_0 is the absorbance of the test sample at 0 min, A_{15} is the absorbance of the test sample at 15 mins and Ac is the absorbance of the control sample at 0 min.

Treatment of cells with phenolic acids and UVA irradiation

Human melanoma G361 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotic solution [1% penicillin (100 units/ml)-streptomycin (100 μg/ml)] at 37°C in humidified air containing 5% CO_2 (P_{CO2} = 40 Torr) (a Forma Scientific CO_2 Water Jacketed Incubator). G361 cells were seeded at 0.5×10^6 cells/well in a 24-well plate for all cell-based assays used to study cellular tyrosinase activity, melanin content, cellular GSH level and oxidant formation. In all cell-based assays, the cells were treated with CA or FA for 30 mins in the PBS before cells were subjected to UVA (320-400 nm) irradiation. The cells were irradiated with UVA light for 5 mins 43 s or 11 mins 26 s to achieve a single UVA dose of 8 J/cm^2 or 16 J/cm^2, respectively.[6] The source of UVA was an xenon arc lamp (Dermalight ultrA1; Hoenle, Germany). The UVA dose of 8 J/cm^2 was applied in our study except for melanin content since such dose did not markedly enhance melanin formation and thus the dose of 16 J/cm^2 was chosen. Moreover, the UVA doses applied in this study were physiologically relevant and did not affect G361 cell viability[6]. The cells were harvested for the assays at 1 h after UV irradiation and cell lysates were prepared using lysis buffer containing 50 mM TrisHCl, 10 mM ethylene diaminetetraacetic acid (EDTA), 1% (v/v) Triton X100, phenylmethylsulfonyl fluoride (PMSF) (100 mg/ml) and pepstatin A (1 mg/ml) in DMSO, and leupeptin (1 mg/ml) in H2O, pH 6.8[1].

Cellular tyrosinase activity assay

Tyrosinase activity was determined by assessing the rate of L-DOPA oxidation to dopachrome as described previously.[1,13] Briefly, cell lysate (90 μl) was loaded onto a 96-well plate and 20 mM L-DOPA (10 μl) was added as the substrate to induce the reaction. Conversion of L-DOPA to dopachrome was measured spectrophotometrically at 475 nm every 10 mins for 1 h at 37°C by a microplate reader. The data have been shown as a percentage of the tyrosinase activity (unit/mg protein) of untreated and non-irradiated control cells (100%).

Melanin content assay

For evaluation of melanin synthesis as described previously,[14] the cell pellets were solubilized in 1 M NaOH and the optical density was measured at 475 nm using a microplate reader. The results were expressed as a percentage of the melanin content (μg/mg protein) of untreated and non-irradiated control cells (100%).

Measurement of intracellular glutathione content

GSH content was evaluated using the fluorescent

probe *o*-phthalaldehyde (OPA) reacting with GSH at pH 8 as previously described[6]. After cells were subjected to UVA irradiation, cells were lysed with 6.5% (w/v) trichloroacetic acid (TCA). The TCA extracts were loaded onto 96-well plates together with buffer (100 mM KH_2PO_4, 10 mM EDTA and 1 mM NaOH) and then OPA (1 mg/ml in methanol). The fluorescence was determined at 350 nm excitation and 420 nm emission. The GSH levels were calculated by comparing with standard curves using known concentrations of GSH. The results have been shown as a percentage of the GSH content (nmol/mg of protein) of the untreated and non-irradiated control cells (100%).

Determination of protein content

The total protein concentration of cell lysates was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Germany).

Determination of intracellular oxidant formation

2′, 7′-Dichlorodihydrofluorescin diacetate (DCFH-DA), a stable and non-fluorescent dye, was used to determine oxidant formation in G361 cells as previously demonstrated.[6] After cells were irradiated with UVA, cells were treated with phenol red-free DMEM containing 5 μM DCFHDA for 1 h. DCF fluorescence was determined for 20 mins at 485 nm excitation and 530 nm emission using a spectrofluorometer. The data have been shown as a percentage of intracellular oxidant formation (relative fluorescence units/RFU) of the untreated and non-irradiated control cells (100%).

Statistical analysis

Data are represented as means ± standard error of the means (SEM) from at least 3 independent experiments. The statistical significance of differences between the control and UVA-irradiated cells was evaluated by Student's *t*-test and between UVA-irradiated and phenolic-treated cells by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

RESULTS

Mushroom tyrosinase inhibitory activity and FR scavenging activity of phenolics

All test phenolics including CA, FA, GA, PA and quercetin (7.5-120 μM) as well as positive control, KA, were shown to exert a dose-dependent protection against mushroom tyrosinase-mediated oxidation of L-DOPA. The IC_{30} value in Table 1 showed a rank order of quercetin ≈ PA > KA ≈ CA ≈ FA > GA for the inhibition of mushroom tyrosinase activity. Additionally, DPPH assay was performed to determine FR scavenging activity of the studied compounds. Based on the IC_{30} values, the rank order of the DPPH radical scavenging activity of the compounds was GA ≈ CA ≈ FA > quercetin > PA ≈ KA.

The effects of CA and FA on UVA-induced tyrosinase activity and melanin synthesis

Since CA and FA exerted powerful FR scavenging activity, they were then chosen for study of their inhibitory effects on UVA-mediated increased melanogenesis in G361 melanoma cells. The cytotoxicity of CA and FA on G361 cells was also assessed by MTT assay. We observed that treatment of the cells with CA and FA up to 120 μM for 24 h did not affect cell viability, indicating that protection by test phenolics against UVA-mediated melanogenesis was not due to reduction in cell numbers.

UVA irradiation led to 49.1 ± 6% ($p < 0.001$) and 36.6 ± 4.2% ($p < 0.001$) induction in tyrosinase activity and melanin content, respectively (Fig 2). However, pretreatment of UVA-irradiated cells with CA and FA

TABLE 1. IC_{30} values of the phenolics for mushroom tyrosinase inhibitory activity and FR scavenging activity.

Test compounds	IC_{30} (μM)	
	Antityrosinase activity	FR scavenging activity
Caffeic acid	$43.09 \pm 2.3^{\#\#\#}$	$14.85 \pm 3.9^{***}$
Ferulic acid	$51.85 \pm 1.7^{\#\#}$	$11.97 \pm 0.6^{***}$
Gallic acid	79.89 ± 6.5	$7.0 \pm 0.5^{***}$
Quercetin	$22.43 \pm 1.5^{*,***}$	$74.23 \pm 9.7^{\#\#\#}$
p-coumaric acid	$22.86 \pm 2.1^{*,***}$	> 120
Kojic acid	$42.78 \pm 6.5^{\#\#\#}$	> 120

For antityrosinase activity: $*p<0.05$ compared with CA and KA. $***p<0.001$ compared with FA and GA. $\#\#p<0.01$; $\#\#\#p<0.001$ compared with GA. For FR scavenging activity: $***p<0.001$ compared with quercetin, PA and KA. $\#\#\#p<0.001$ compared with PA and KA.

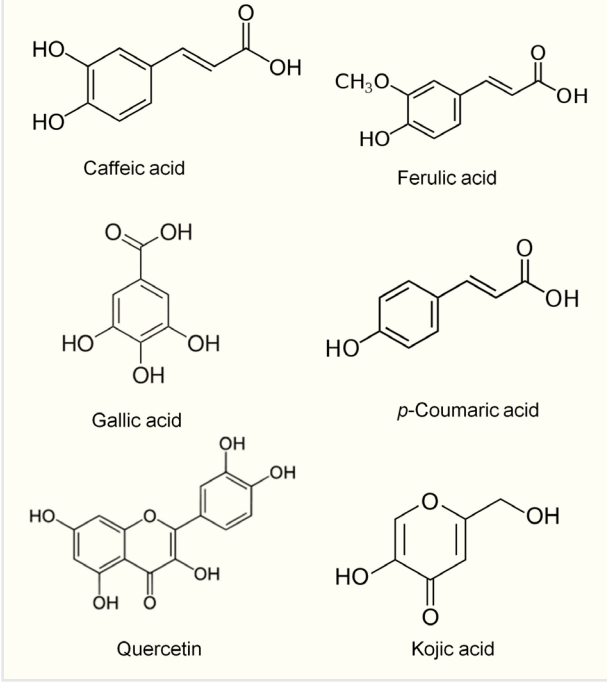

Fig 1. Chemical structure of test phenolics.[7-12]

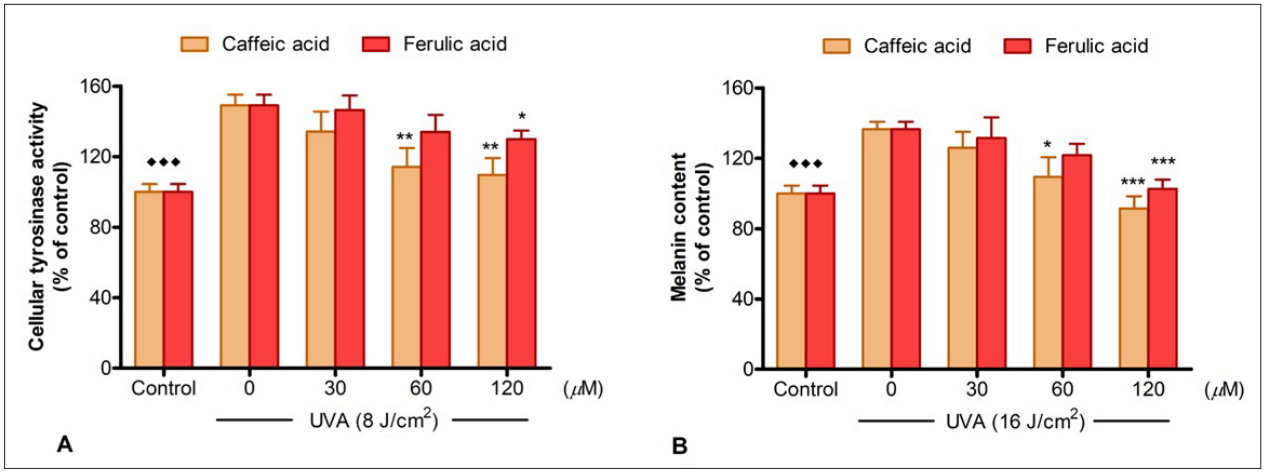

Fig 2. Protection by CA and FA against UVA-mediated melanogenesis in G361 cells. Cellular tyrosinase activity (A) and melanin formation (B) induced by a single dose of UVA at 8 or 16 J/cm^2, respectively. ♦♦♦ $p < 0.001$ compared with UVA-irradiated cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with untreated cells exposed to UVA.

resulted in a substantial decline in tyrosinase activity (Fig 2A) and melanin content (Fig 2B) in a concentration-dependent manner. Our data also showed that CA had a greater antimelanogenic effect than that of FA since a lower dose of CA was required to prevent induction of tyrosinase activity and melanin content in irradiated cells.

The effects of CA on UVA-induced oxidant formation and GSH content

Protection by CA against oxidant formation and GSH loss in UVA-irradiated G361 cells was then assessed because it was more potent than FA in inhibiting UVA-mediated melanogenesis. Irradiation of cells by a UVA dose of 8 J/cm^2 produced a 67.6 ± 7.2% (p<0.001) augmentation of oxidant formation compared to non-UVA-irradiated cells, although pretreatment with CA led to a dose-dependent decrease in cellular oxidant level in response to UVA irradiation (Fig 3A). In addition, while exposure of the cells to UVA irradiation (8 J/cm^2) substantially reduced GSH content by 45.98 ± 6.2% ($p < 0.001$) compared to non-irradiated cells, pretreatment with CA significantly blocked GSH depletion in a concentration-dependent manner (Fig 3B).

DISCUSSION

Screening for antityrosinase properties of various phenolic compounds possessing antioxidant actions has gained a lot of attention in order to develop putative whitening agents. Several reports have suggested that compounds yielding antioxidant properties could also serve as potential antityrosinase agents capable of blocking melanin synthesis.[6,15] GA, CA and FA are phenolic acids naturally present in a variety of medicinal plants used for skin problems including hyperpigmentation, probably, through their antioxidant actions.[16-19] Therefore, we carried out comparative *in vitro* evaluation of antityrosinase and antioxidant activities of several natural phenolics. By using cell-free system models, our data showed that protection by the test phenolics against mushroom tyrosinase activity were not correlated with their FR scavenging activities because GA possessed lower inhibitory effects

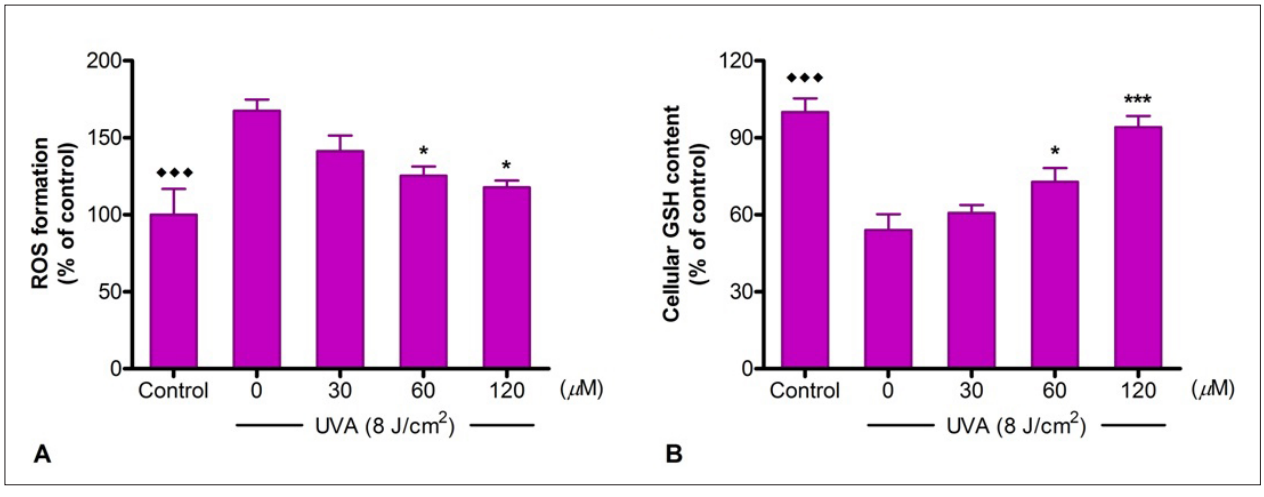

Fig 3. Protection by CA against UVA-mediated oxidant formation and GSH loss in G361 cells. Cellular oxidant formation (A) and GSH content (B). ♦♦♦ $P < 0.001$ compared with UVA-irradiated cells. *p<0.05; **p<0.01; ***p<0.001 compared with untreated cells exposed to UVA.

on mushroom tyrosinase than quercetin, PA, CA and FA, while it yielded greater FR scavenging activity than quercetin and PA. Furthermore, CA and FA were demonstrated to possess antityrosinase and antioxidant activity, while our findings in the mushroom tyrosinase assay did not relate to that observed in cellular tyrosinase study. CA and FA were shown to have comparable inhibitory activities against mushroom tyrosinase and DPPH free radicals in a cell-free system, whereas CA provided greater protective effect than FA on UVA-mediated melanogenesis in G361 cells. Furthermore, our previous study showed that GA, which yielded lower abilities to inhibit mushroom tyrosinase than CA and FA in this study, was more effective than CA and FA in inhibiting UVA-mediated cellular melanogenesis as a lower concentration of GA (15 μM) than that of CA (60 and 120 μM) and FA (120 μM) was required to reduce tyrosinase activity and melanin content in UVA-irradiated G361 cells.[19]

Tyrosinase catalyzes two distinct rate-limiting steps in melanin biosynthesis; the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to DOPAquinone, a highly reactive o-quinone, readily converted to dopachrome eventually leading to melanin production involving a series of complex chemical reactions including oxidation-reduction reactions.[20] Antityrosinase properties of depigmenting compounds may be attributed to different actions including competitive inhibition at the copper catalytic site of tyrosinase and non-competitive inhibition against L-DOPA oxidation, reduction of *o*-quinone to prevent dopachrome formation and detoxification of ROS involved in melanin formation.[21,22] Therefore, compounds that are effective in inhibition of mushroom tyrosinase activity may not serve as powerful FR scavengers. Well-known tyrosinase inhibitors including arbutin, hydroquinone and KA are competitive tyrosinase inhibitors, but are not represented as strong FR scavengers. Our study confirmed that while KA had a greater ability than GA to inhibit mushroom tyrosinase activity, its FR scavenging activity was weaker than that of GA. Therefore, developing dietary phenolics as candidate and effective depigmenting agents is promising since they could have a potential to interfere with various steps of melanin formation.

UVA irradiation-mediated GSH depletion has been proposed to be responsible for abnormal melanogenesis.[6,19] Our study demonstrated that the antimelanogenic effect of CA was associated with its ability to restore redox balance through upregulation of GSH content in the irradiated cells. Indeed, inhibitory activity of phenolics against mushroom tyrosinase appeared to be uncorrelated with that against cellular melanogenesis. It is probably because of different depigmenting mechanisms involving various targets such as direct inhibition of tyrosinase activity, transcriptional and/or translational regulations of tyrosinase, chemical reactions involved in melanin formation, melanosome transfer and/or distribution, melanocyte viability and abilities of a compound to permeate into the cells, these account for their intracellular availability and activity.[23-25] Our observations were consistent with previous studies reporting that while citrus fruit extract exhibited a weak inhibitory activity against mushroom tyrosinase, it was capable of protection against melanogenesis in both cultured B16 melanoma cells and brown guinea pig skin exposed to UVB.[26]

Therefore, suppression of melanogenesis cannot be attributed to inhibitory activity against tyrosinase or antioxidant activity alone. Furthermore, model systems and cell types employed should be taken into consideration when assessing antityrosinase properties of depigmenting agents.

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