SUPPLEMENTARY MATERIAL

(−)-β-Homoarginine anhydride, a new antioxidant and tyrosinase inhibitor, and further active components from *Trichosanthes truncata*

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ABSTRACT

One new amino acid derivative, \((-\)-\(\beta\)-homoarginine anhydride 1, as well as nine known compounds were isolated from *Trichosanthes truncata*. The structures of the isolates were elucidated by spectroscopic methods. Among them, compounds 5 and 11 could notably dose-dependently inhibit ROS productions in HaCaT keratinocyte cells without cytotoxicity in the concentration range of 0.2 to 20 \(\mu\)M. In cell-free mushroom tyrosinase assay, compounds 1–5, 10 and 11 had more potential anti-tyrosinase activities with IC\(_{50}\) values of 106.9–255.6 \(\mu\)M than arbutin that were similar to predicted values of binding affinity calculated by molecule docking. The most active 2 had hydrogen bonds (Ser77, Glu309, Phe454) and electrostatic charges (Glu309, Glu248) interactions with mushroom tyrosinase, respectively. Our data manifested that *T. truncata* and its components are potentially to be developed as anti-aging and whitening agents for skin disorders.

KEYWORDS: *Trichosanthes truncata*; Cucurbitaceae; Antioxidant; Tyrosinase inhibition
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Experimental in detail

1. General experimental procedure

1D and 2D NMR spectra were taken on Bruker 500 MHz FT-NMR. The chemical shift ($\delta$) values are reported in ppm (part per million) with pyridine-$d_5$ and methanol-$d_4$ as internal standards, and coupling constants ($J$) are in Hz. Low- and high-resolution ESIMS and EIMS were measured on a Bruker Daltonics Esquire HCT ultra high capacity trap mass spectrometer and an Orbitrap mass spectrometer (LTQ Orbitrap XL and Q Exactive Plus, Thermo Fisher Scientific), respectively. Optical rotation and IR spectrum were recorded on JASCO-P-2000 polarimeter (cell length 10 mm) and Shimadzu model IR Prestige-21 Fourier-transform infrared spectrophotometer, respectively. TLC was performed on Merck DC Kieselgel 60 $F_{254}$ and DC Kieselgel 60 RP-18 $F_{254}s$, spots were recognized under ultraviolet light at 254 and 356 nm and then stained by spraying with 5% $H_2SO_4$ in MeOH before heating on hot plate. For column chromatography, silica gel (siliaFlash P60 70–230, 230–400 mesh), RP-18 (LiChroprep RP-18, 230–400 mesh), Sephadex™ LH-20 (GE Healthcare, Uppsala, Sweden), Diaion® HP-20 (Supelco™, Bellefonte), and Amberlite XAD7 (20–60 mesh, SIGMA) were used. Mushroom tyrosinase (lyophilized powder, $\geq$1000 unit/mg solid), $\alpha,\alpha$-diphenyl-$\beta$-picrylhydrazyl (DPPH), and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma.

2. Extraction and isolation

The roots of $T. truncata$ (40.0 g) were powdered and extracted three times with MeOH, EtOH, and water (200 mL $\times$ 3) at room temperature to obtain MeOH (MTR, 1.3 g), EtOH (ETR, 0.5 g), and water (WTR, 4.0 g) extracts, respectively. Additionally, the material (40.0 g) was also extracted once with hot water to give BTR extract (4.1 g). Among above-mentioned four crude extracts, MTR showed potential bioactive properties and was selected as a candidate to clarify its active components.

The MTR extract (120.0 g) was prepared from $T. truncata$ (2.4 kg). MTR was then partitioned
with EtOAc and water (1:1, v/v), then the water elute was further partitioned with n-BuOH to obtain EtOAc- (MTRE, 15.0 g), n-BuOH- (MTRB, 25.0 g), and water-soluble (MTRW) extracts, respectively. Fractionation of MTRE was conducted by open column chromatography on silica gel (770 mL; 0.063–0.200 mm) using gradients of hexene–CH₂Cl₂ to CH₂Cl₂–MeOH to yield 14 fractions.

Fraction 9 (1.7 g) was subjected to an XAD2 column (198 mL) using MeOH–water gradient system (60–100%) to afford six subfractions. Subfraction 9-6 (641.4 mg) was purified by silica gel column (127 mL; 40–60 µm) eluted with hexane–CH₂Cl₂–MeOH (1:1:0, 0:1:0, 0:20:1) to give nine subfractions, the eighth one contained a mixture 8 (108.7 mg).

Fraction 10 (1.9 g) was fractionated on Sephadex LH-20 (377 mL; CH₂Cl₂–MeOH, 1:1) to get 13 subfractions. Subfraction 10-7 (58.5 mg) was further purified by column chromatography (127 mL; 40–60 µm) with a gradient of CH₂Cl₂–MeOH (70:1 to 15:1) to give 11 subfractions. Subfraction 10-7-7 (50.7 mg) was purified by silica gel column (59 mL; 40–60 µm) eluted with hexane–acetone (6:1) to obtain five subfractions. Compound 9 (7.0 mg) was crystallized from the fourth and fifth subfractions. Subfraction 10-10 (105.6 mg) was subjected to a silica gel column (40 mL; 40–60 µm) eluted with hexane–EtOAc (3:2) to obtain five subfractions. The fourth subfraction was further purified by silica gel column (82 mL; 40–60 µm) with hexane–EtOAc (3:1) to get 5 (12.9 mg), and the fifth subfraction was subjected to a RP-18 column (82 mL; 40–63 µm) to get nine subfractions, the first one was then purified by silica gel column (82 mL; 40–60 µm) with hexane–EtOAc (3:1) to get 2 (16.8 mg).

Fraction 11 (4.0 g) was separated on Sephadex LH-20 (1140 mL; CH₂Cl₂–MeOH, 1:1) to get eight subfractions and compound 7 (163.0 mg) was crystallized from the fourth subfraction. Subfraction 11-4 was then subjected to a silica gel column (160 mL; 40–63 µm) with a gradient of CH₂Cl₂–MeOH (30:1 to 4:1) to give 14 subfractions, the third one (64.5 mg) was purified by RP-18 column (74 mL; 40–63 µm) with 60% MeOH to afford 10 (2.2 mg). Subfraction 11-5 was subjected to a silica gel column (113 mL; 40–63 µm) with a gradient of CH₂Cl₂–MeOH (30:1 to
5:1) to give 10 fractions, the seventh fraction (32.0 mg) was purified by silica gel column (43 mL; 40–63 µm) to give 6 (6.6 mg).

MTRB (25.0 g) was fractionated through a Diaion HP-20 column (1040 mL) and eluted with H₂O, 50% MeOH, MeOH, and acetone, respectively. Column chromatography of the 50% MeOH-eluted portion (8.3 g) on XAD7 gel with a gradient of 40 to 100% MeOH gave four fractions. Fraction 1 (6.7 g) was subjected to a RP-18 column (550 mL; 40–60 µm) with MeOH–H₂O (1:3, 1:2, 1:1) to give eight fractions. Fraction 1-1 (1.1 g) was subjected to a silica gel column (255 mL; 40–60 µm) with CH₂Cl₂–MeOH (10:1, 8:1, 5:1, 2:1) to afford 17 fractions and compound 1 (11.0 mg). Fraction1-1-6 (15.9 mg) was further purified by silica gel column (20 mL; 40–60 µm) eluted with hexane–EtOAc (1:7) to afford 3 (3.6 mg). Fraction 1-1-8 (14.9 mg) was subjected to a silica gel column (16 mL; 40–60 µm) with CH₂Cl₂–MeOH (20:1 to 10:1) to give 4 (4.8 mg).

3. Acid hydrolysis

Compound 7 (33.0 mg) was heated in 10 mL of 1N HCl and 1,4-dioxane (1:1) at 110°C for 6 h. The reaction mixture was concentrated in vacuo, then partitioned with CH₂Cl₂–H₂O to get steroid 11 (25.4 mg) identified by NMR spectroscopy.

4. DPPH free radical scavenging assay (Eseyin et al. 2018; Jorge et al. 2015; Lu and Ko 2016)

The assay solution consisted 150 µL of DPPH ethanol solution (250 µM) and 50 µL of test samples with different concentrations. The mixtures were incubated in dark at room temperature for 20 min. The absorbance of mixture was carried out by using a micro plate reader (SPECTROstar® Nano, BMG LABTECH) at 517 nm. Ascorbic acid was used as the positive control. All the measurements were performed in triplicate. The free radical scavenging activities were calculated as follows:

Scavenging rate (%) = (1–A_{sample}/A_{0}) ×100; A_{sample} is the absorbance of the sample solution at
steady state and $A_0$ is the absorbance of DPPH solution without sample addition.

5. Cytotoxicity

The cytotoxicities of HaCaT cells for test compounds were analyzed with MTT as described previously protocol with slight modifications (Cholia et al. 2017). Cells were cultivated in Dulbecco’s Modified Eagle Medium (DMEM; GIBCO Invitrogen corporation, NY, USA). The 100 μL of MTT reagent (Thermo Scientific) (0.5 mg/mL) was added to each well and then the plates were incubated for 1 h in the dark at 37 °C. The formazan product formed was dissolved in acidified DMSO and cell viabilities were measured by using a micro plate reader (SPECTROstar® Nano, BMG LABTECH) at 570 nm.

6. Anti-ROS induced in keratinocyte cell assay

All isolates were assayed by the reported methods (Bryan and Grisham 2007; Cholia et al. 2017; Kim and Yoo 2016) with some modifications. HaCaT cells were treated with 10 μM H$_2$O$_2$ for 3 h and then with test samples for 16 h. The ROS indicator, CM-H$_2$DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) (10 μM) was added after removing the mediums and incubated for 1 h in dark room and detected the fluorescence intensity by using cytofluorometer (CYTATION 5, BioTek®) recorded at excitation/emission 485/535 nm.

7. Mushroom tyrosinase inhibition assay

This assay was performed by methods described previously with some modifications (Ferro et al. 2018; Lu and Ko 2016; Seo et al. 2010). Briefly, 40 μL of mushroom tyrosinase (EC 1.14.18.1) (Sigma Chemical Co.) buffer solution (500 U/mL), 30 μL of the sample solution with different concentrations, and 50 μL of L-DOPA (3,4-dihydroxyphenylalanine) (5 mM) were added in the wells of a 96-well micro plate, respectively. The plate was further incubated at room temperature in dark for 20 min. The absorbance of mixtures was determined at 475 nm using a micro plate
reader (SPECTROstar® Nano, BMG LABTECH). Arbutin was used as the positive control. All the measurements were performed in triplicate. The tyrosinase inhibition activities were calculated as follows:

\[
\text{inhibition} \% = \frac{[(A - B) - (C - D)]}{A - B} \times 100
\]

A: Abs of the enzyme and solution without sample
B: Abs of solution without sample
C: Abs of the substrate, the test sample solution, and the enzyme
D: Abs of the substrate, the test sample solution

8. Melanin content and tyrosinase activity assay in B16F10 cells

B16F10 murine melanoma cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM; GIBCO Invitrogen corporation, NY, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in an incubator with 5% CO₂. Cells were harvested by trypsinization. Cell viability assay was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) agent. The melanin contents in B16F10 cells were assayed with methods previously reported (Chiang et al. 2014; Kuo et al. 2015) and determined by an ELISA reader (Tecan, Grodig, Austria) at 405 nm.

9. Molecular docking

To calculate the binding affinity between compounds and mushroom tyrosinase, the molecular docking was performed by PyRx 0.8 (Dallakyan and Olson 2015) running AutoDock Vina 1.1.2 package (Trott and Olson 2010). The energy minimization of compound structures was performed with MMFF94 force field using conjugate gradients algorithm by running Open Babel (O'Boyle et al. 2011) in PyRx 0.8. The protein structure of mushroom tyrosinase (PDB ID: 5M6B) (Pretzler et al. 2017) was obtained from the Protein Data Bank (https://www.rcsb.org/) and the two cocrystal copper ions in active site of mushroom tyrosinase were removed before docking calculation. To identify the grid box location and size as the search space for docking calculation, the center of
grid box was set at the center between two copper ions in the active site of mushroom tyrosinase and the size is big enough to include active site for docking, according to previous studies (Pretzler et al. 2017), so the grid dimensions of x, y, and z axis were set 25.51, 39.11, and 26.29 Å, respectively, and the origin coordinates were set at $x = -34.03$, $y = -17.18$, $z = 14.67$. The results of docking simulation were visualized by BIOVIA Discovery Studio 4.5 Visualizer (Dassault Systemes, BIOVIA Corp., San Diego, CA, USA).

10. Molecular preparing

The 2D and 3D structures of compounds were constructed by ChemBioDraw Ultra 11.0 and ChemBio3D Ultra 11.0 (CambridgeSoft Corp.), respectively.

Figure S1. Anti-ROS induced in HaCaT keratinocyte cells activities (A) and cytotoxicities toward HaCaT cells (B) of MTR, ETR, WTR, and BTR extracts.
Figure S2. DPPH-scavenging activity data (A) and tyrosinase inhibition data (B) of MTR, ETR, WTR, and BTR extracts

Figure S3. Anti-ROS induced in HaCaT keratinocyte cells activities (A) and cytotoxicities toward HaCaT cells (B) of MTRE, MTRB, and MTRW crude fractions.

Figure S4. DPPH-scavenging activity data (A) and tyrosinase inhibition data (B) of MTRE, MTRB, and MTRW crude fractions.
**Figure S5.** The key HMBC and $^1$H-$^1$H COSY correlations of compound 1.

**Table S1.** $^1$H-NMR and $^{13}$C-NMR data for compound 1 (500, 125 MHz in C$_5$D$_5$N).$^a$

| position | $\delta_H$ (J, Hz) | $\delta_C$ |
|----------|--------------------|-----------|
| 1, 1'    |                    | 171.5     |
| 2, 2'    | 2.29 dd (16.0, 13.0) | 37.8      |
|          | 2.71 dd (16.0, 13.0) |
| 3, 3'    | 3.53 m             | 53.5      |
| 4, 4'    | 1.29 m             | 33.1      |
|          | 1.89 m             |
| 5, 5'    | 1.56 m             | 23.2      |
|          | 1.69 m             |
| 6, 6'    | 3.45 m             | 44.7      |
|          | 3.57 m             |
| 8, 8'    |                    | 152.0     |

$^a$ m: multiplet signals.
Figure S6. The structures of compounds 2–11.

Figure S7. $^1$H NMR (500 MHz, C$_5$D$_5$N) of compound 1
Figure S8. $^{13}$C NMR (125 MHz, C$_5$D$_5$N) of compound 1

Figure S9. HSQC NMR (500 MHz, C$_5$D$_5$N) of compound 1
Figure S10. HMBC NMR (500 MHz, C₅D₅N) of compound 1

Figure S11. COSY NMR (500 MHz, C₅D₅N) of compound 1
Figure S12. (A) ESI-MS and (B) HR-ESI-MS spectra of compound 1
**Figure S13.** IR spectrum of compound 1

**Figure S14.** Cytotoxicities toward HaCaT cells of compounds 1–11.
Figure S15. Anti-ROS generation induced in HaCaT keratinocyte cells of compounds 1–11.

Figure S16. Effects of compounds 1, 5, and 11 on H₂O₂-induced ROS production in HaCaT keratinocyte cells at the concentrations of 4 and 20 μM. The intracellular levels of ROS were imaged on fluorescence microscopy (scale gauge: 1000 μm).
### Table S2. Tyrosinase inhibition activities of compounds 1–11.

| Compound | Tyrosinase inhibition (μM) |
|----------|-----------------------------|
| 1        | 175.2 ± 32.4                |
| 2        | 106.9 ± 15.5                |
| 3        | 249.6 ± 29.1                |
| 4        | 237.0 ± 50.3                |
| 5        | 230.7 ± 41.7                |
| 6        | >300                        |
| 7        | >300                        |
| 8        | >300                        |
| 9        | >300                        |
| 10       | 147.4 ± 17.5                |
| 11       | 255.6 ± 29.5                |
| Arbutin  | 285.5 ± 73.8                |

### Table S3. Predicted binding affinity of compounds 1–11 on mushroom tyrosinase.

| Compound | Binding affinity (kcal/mol) |
|----------|-----------------------------|
| S,S-1    | -6.9                        |
| R,R-1    | -6.8                        |
| 2        | -5.7                        |
| 3        | -4.7                        |
| 4        | -5                          |
| 5        | -5.1                        |
| 6        | -5                          |
| 7        | 18.8                        |
| 8a       | 4.3                         |
| 8b       | 4.2                         |
| 9        | 21.6                        |
| 10       | -5.5                        |
| 11       | 4.2                         |
| Arbutin  | -4.1                        |
Figure S17. The simulation of compounds S,S-1 (A) and R,R-1 (B) docking into the active site of mushroom tyrosinase (PDB ID: 5M6B). Hydrogen bonds (dashed green line) and electrostatic charge (dashed orange line) interactions into active site of tyrosinase, respectively.

Figure S18. Cytotoxicity toward B16F10 cells and melanin content inhibition of compound 2 under different concentrations.
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