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Potent inhibitors of tyrosinase activity from *Citrullus colocynthis* Schrad. (Cucurbitaceae)

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Abstract

A new fatty acid glucoside, citrulloside (**1**) along with nine known compounds were isolated from the fruit extracts of *Citrullus colocynthis* Schrad. (Cucurbitaceae). The structures of the compounds were elucidated by spectroscopic analysis, mainly 1D and 2D NMR and comparing their physical (mp) and spectroscopic data with those reported in the literature. Compounds **1-5** were tested for their enzyme tyrosinase activity. The results showed that cucurbitacin E (**2**) (IC_{50}= 1.99 μM) exhibited a more potent inhibition than the standard inhibitors kojic acid and L-mimosine.

Keywords: *Citrullus colocynthis*, Tyrosinase inhibition, Citrulloside, Cucurbitacin

Introduction

Citrullus is a small genus with 4 or 5 species of creeping or climbing herbs distributed in tropical Africa and Asia. The constituents of Cucurbitaceae were known for a long time for their purgative activity. The tumour inhibition properties of the cucurbitacins are well known (Kupchan et al. 1967). Dried unripe fruit pulp constitutes the drug, which is a very strong laxative. Also used as an antirheumatic, antihelmintic and as a remedy for skin infection. Roots have purgative properties and are used in jaundice, rheumatism and urinary diseases (Keraudren 1966). Tyrosinase (monophenol monooxygenase, E.C. 1.14.18.1) is a multifunctional copper-containing enzyme widely distributed in plants and animals. It catalyses two reactions involving molecular oxygen in the melanin biosynthesis pathway: the oxidation of monophenols to *O*-quinones (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). These quinones are highly reactive and tend to polymerise spontaneously to form brown pigments of high molecular weight (melanins), which determine the colour of mammalian skin and hair (Seo et al. 2003). Quinones can also react with amino acids and proteins and thus enhance the development of brown color. Therefore, tyrosinase inhibitors should be clinically useful for the treatment of some dermatological disorders such as melasma, age spots and sites of actinic damage, associated with melanin hyperpigmentation. Tyrosinase inhibitors have become increasingly important in medication (Seo et al. 2003) and in cosmetics for whitening and to prevent hyperpigmentation by inhibiting enzymatic oxidation (Maeda and Fukuda 1991). In addition, tyrosinase is known to be involved in the molting process of insects and adhesion of marine organisms (Shiino et al. 2001). Cucurbitacins and its glycosides have been reported as cytotoxic and anti-tumour in experimental

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tumours (Kupchan et al. 1967). They produced growth inhibition of solid tumours *in vivo* as well as characteristic morphological changes of Ehrlic ascites tumour cells i*n vitro*. Cucurbitacins have also been reported as inhibitors of cell adhesion (Laszlo et al. 1994). A number of naturally occuring tyrosinase inhibitors consist of a phenol structure or of metal chelating agents (Pifferi and Baldassari 1974, Mayer and Harel 1979, Passi and Nazzaro-Porro 1981, Mayer 1987). In the present study, the isolation and characterization of a new compound to which the trivial name citrulloside (**1**) has been assigned are described. In a continuing search for tyrosinase inhibitors from plants, cucurbitacin E (**2**), 2-O-β-D-glucopyranosyl cucurbitacin E (**3**), 2-O-β-D-glucopyranosyl cucurbitacin I (**4**) and 2-O-β-D-glucopyranosyl cucurbitacin L (**5**) isolated from the EtOAc and $CHCl_3$ fruit extracts of *Citrullus colocynthis* were found to inhibit oxidation of L-DOPA by mushroom tyrosinase. This is the first time these compounds are studied in this context.

Materials and Methods

General methods

Optical rotations were measured on Schmid Haensch Polartronic D. EI and HREI MS were measured on Varian MAT 311A and Jeol-HX 110 mass spectrometers; *m/z* (Rel Int.). The ^{1}H-NMR, ^{13}C-NMR, COSY, HMQC and HMBC spectra were recorded on Brucker AMX 400 MHz and AMX 500 MHz spectrometers. Chemical shifts are in ppm (δ) relative to $SiMe_4$ as standard and coupling constants are in Hz. Melting points were measured on a YANACO melting point apparatus. Column chromatography (CC) was carried out on silica gel (70-230 mesh). Thin layer chromatography (TLC) was performed on pre-coated silica gel plates (DC Alufolien to F_{254} of Merck) and spots were detected by UV at 254 and 366 nm and also by using ceric sulphate spraying reagents.

Plant Material

Fruits of *Citrullus colocynthis* were purchased from Karachi market on October 2001.

Extraction and isolation

Air-dried and pulverised fruits of *Citrullus colocynthis* (10 kg) were soaked in MeOH (9 days) and the concentrated methanol extract was partitioned with *n*-hexane, $CHCl_3$ and EtOAc. The EtOAc extract (330 g) was subjected to vacuum liquid chromatography (VLC) (silica gel, 1 kg) using a gradient of $CHCl_3$ to MeOH. The 20% MeOH-$CHCl_3$ VLC fraction (80 g) which was subjected to further VLC (silica gel, *n*-hexane-acetone, 60:40) and the 10% MeOH-$CHCl_3$ VLC fraction (5 g) were subjected to further purification by column chromatography (silica gel, eluent *n*-hexane-EtOAc, 90:10) and both yielded β-stigmasterol (40 mg), *p*-hydroxybenzyl alcohol (20 mg), cucurbitacin E (**2**) (100 mg) and compound **1** named citrulloside (50 mg). The 40% MeOH-$CHCl_3$ VLC fraction yielded after column chromatography (silica gel, $CHCl_3$-MeOH, 90:10) *p*-hydroxybenzoic acid (10 mg) and 2-O-β-D-glucopyranosyl cucurbitacin E (**3**) (1 g). The 50% MeOH-$CHCl_3$ VLC fraction yielded after HPLC 2-O-β-D-glucopyranosyl cucurbitacin I (**4**) (100 mg) and 2-O-β-D-glucopyranosyl cucurbitacin L (**5**) (50 mg). The $CHCl_3$ extract 40 g was subjected to column chromatography over silica gel using a gradient of *n*-hexane to $CHCl_3$ and $CHCl_3$ to MeOH. The 60% $CHCl_3$-*n*-hexane fraction yielded *p*-hydroxybenzaldehyde (20 mg) and *p*-methoxymethyl-phenol (25 mg).

Citrulloside **1**, non-crystalline solid, mp 176 °C (MeOH). $[\alpha]^{25}_D + 8°$ (*c* 0.2, MeOH). IR bands (KBr, cm^{-1}): 3373, 2919, 2850, 1725, 1642 cm^{-1}. UV max (MeOH): 204 (log ε 3.98) nm. ^{1}H-NMR (500 MHz, CD_3OD): see Table 1. ^{13}C-NMR (125 MHz, CD_3OD): see Table 1. Positive FAB-MS *m/z* (rel. Int. %): 715 (7), 697 (10), 645 (5), 553 (10), 461 (15), 369 (10), 277 (100), 259 (20), 185 (100). FABHRMS *m/z*: 715.5418 $[M + H]^+$ Calc. for $C_{40}H_{74}O_{10}$ 715.4838.

Cucurbitacin E **2**, white amorphous powder, mp 230-235 °C (pyridin). IR bands (KBr, cm^{-1}): 3440, 1720, 1650, 1630. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 6.31(d, 1H, J = 2.2 Hz, H-1), 5.68 (t, 1H, J = 2.2 Hz, H-6), 2.23 (dd, 2H, J = 8.0 and 19.3 Hz, H-7), 3.75 (brs, 1H, H-10), 1.16 (d, 1H, J = 12.8 Hz, H-12a), 1.95 (d, 1H, J = 12.8 Hz, H-12b), 3.37 (d, 1H, J = 14.0 Hz, H-15a), 2.94 (d, 1H, J = 14.5 Hz, H-15b), 5.13 (dd, 1H, J = 7.5 and 12.0 Hz, H-16), 2.01 (d, 1H, J = 8.6 Hz, H-17), 1.12 (s, 3H, H-18), 1.20 (s, 3H, H-19), 1.45 (s, 3H, H-21), 7.35 (d, 1H, J = 15.8 Hz, H-23), 7.40 (d, 1H, J = 15.8 Hz, H-24), 1.30 (s, 3H, H-26), 1.71 (s, 3H, H-27), 1.60 (s, 3H, H-28), 1.51 (s, 3H, H-29), 1.54 (s, 3H, H-30), 10.62 (s, 1H, OH-2), 6.22 (d, 1H, J = 4.6 Hz, OH-16), 6.37 (s, 1H, OH-20), 1.45 (s, 3H, $C\underline{H}_3CO$). ^{13}C-NMR (125 MHz, pyridin-d_5) δ: 115.8 (C-1), 147.3 (C-2), 198.3 (C-3), 48.6 (C-4), 120.5 (C-5), 122.5 (C-6), 24.0 (C-7), 42.1 (C-8), 48.6 (C-9), 35.2 (C-1), 213.7 (C-11), 49.3 (C-12), 79.7 (C-13),51.0 (C-14), 46.8 (C-15), 70.7 (C-16), 59.8 (C-17), 20.1 (C-18), 18.4 (C-19), 79.7 (C-20), 25.6 (C-21), 204.3 (C-22), 137.8 (C-23), 147.3 (C-24), 79.8 (C-25), 26.5 (C-26), 26.2 (C-27), 20.6 (C-28), 28.0 (C-29), 20.8 (C-30), 21.7 ($\underline{C}H_3CO$), 169.8

($CH_3\underline{C}O$). EIMS *m/z* (rel. Int. %) 496 (3), 164 (15), 96 (100), 81 (7), 69 (7). HREIMS *m/z*: 556.2827 (calcd. for $C_{32}H_{44}O_8$, 556.2824).

2-O-*β*-D-glucopyranosyl cucurbitacin E **3**, yellow amorphous powder. mp 150-152°C (MeOH). IR bands (KBr, cm^{-1}): 3440, 1720, 1685, 1640. ^{1}H-NMR (400 MHz, acetone-d_6) δ: 6.10 (d, 1H, J = 2.4 Hz, H-1), 5.83 (t, 1H, J = 2.0 Hz, H-6), 2.55 (dd, 2H, J = 8.0 and 19.3 Hz, H-7), 3.67 (brs, 1H, H-10), 1.85 (d, 1H, J = 8.0 Hz, H-12a), 2.09 (d, 1H, J = 12.8 Hz, H-12b), 3.37 (d, 1H, J = 14.0 Hz, H-15a), 2.95 (d, 1H, J = 8.0 Hz, H-15b), 4.58 (d, 1H, J = 7.6 Hz, H-16), 2.01 (d, 1H, J = 8.6 Hz, H-17), 0.88 (s, 3H, H-18), 1.00 (s, 3H, H-19), 1.29 (s, 3H, H-21), 6.89 (d, 1H, J = 15.7 Hz, H-23), 6.95 (d, 1H, J = 15.7 Hz, H-24), 1.26 (s, 3H, H-26), 1.56 (s, 3H, H-27), 1.53 (s, 3H, H-28), 1.40 (s, 3H, H-29), 1.29 (s, 3H, H-30), 1.99 (s, 3H, $C\underline{H}_3CO$), 4.63 (d, 1H, J = 7.5 Hz, H- 1′), 3.30 (dd, 1H, J = 7.5 and 9.0 Hz, H-2′), 3.18 (dd, 1H, J = 7.5 and 9.5 Hz, H-3′), 3.35 (dd, 1H, J = 9.5 and 9.5 Hz, H-4′), 3.98 (m, 1H, H-5′), 4.04 (dd, 1H, J = 2.2 and 12.1 Hz, H-6a′), 3.86 (dd, 1H, J = 3.6 and 12.5 Hz, H-6b′). Negative FAB-MS *m/z* (rel. Int. %): 717 (18), 496 (10), 311 (45), 183 (30), 163 (40).

2-O-*β*-D-glucopyranosyl cucurbitacin I **4**, white amorphous powder. mp 229-230°C (MeOH). IR bands (KBr, cm^{-1}): 3440, 1720, 1650, 1630. ^{1}H-NMR (500 MHz, MeOD) δ: 6.10 (d, 1H, J = 2.4 Hz, H-1), 5.83 (t, 1H, J = 2.0 Hz, H-6), 2.40 (dd, 2H, J = 8.6 and 19.7 Hz, H-7), 3.68 (brs, 1H, H-10), 1.87 (dd, 1H, J = 8.7 and 14.7 Hz, H-12a), 2.05 (d, 1H, J = 8.0 Hz, H-12b), 2.66 (d, 1H, J = 14.7 Hz, H-15a), 2.60 (d, 1H, J = 7.2 Hz, H-15b), 4.51 (d, 1H, J = 7.2 Hz, H-16), 0.93 (s, 3H, H-18), 1.00 (s, 3H, H-19), 1.29 (s, 3H, H-21), 6.82 (d, 1H, J = 15.7 Hz, H-23), 6.96 (d, 1H, J = 15.7 Hz, H-24), 1.26 (s, 3H, H-26), 1.40 (s, 3H, H-27), 1.39 (s, 3H, H-28), 1.40 (s, 3H, H-29), 1.32 (s, 3H, H-30), 4.62 (d, 1H, J = 7.2 Hz, H- 1′), 3.35 (dd, 1H, J = 7.5 and 9.0 Hz, H-2′), 3.31 (dd, 1H, J = 7.5 and 9.5 Hz, H-3′), 3.36 (dd, 1H, J = 9.5 and 7.8 Hz, H-4′), 3.53 (m, 1H, H-5′), 4.04 (dd, 1H, J = 2.2 and 12.1 Hz, H-6a′), 3.86 (dd, 1H, J = 3.6 and 12.2 Hz, H-6b′). Negative FAB-MS *m/z* (rel. Int. %): 675 (30), 659 (20), 459 (10), 367 (22), 275 (100), 165 (60), 149 (85).

2-O-*β*-D-glucopyranosyl cucurbitacin L **4**, white amorphous powder. mp 233-235°C (MeOH). IR bands (KBr, cm^{-1}): 3440, 1720, 1650, 1630. ^{1}H-NMR (400 MHz, MeOD) δ: 6.03 (d, 1H, J = 2.4 Hz, H-1), 5.83 (brs, 1H, H-6), 2.38 (dd, 1H, J = 8.6 and 19.7 Hz, H-7a), 1.93 (m, 1H, H-7b), 1.95 (m, 1H, H-8), 3.66 (brs, 1H, H-10), 2.48 (d, 1H, J = 14.7 Hz, H-12a), 3.24 (d, 1H, J = 14.8 Hz, H-12b), 1.37 (m, 1H, H-15a), 1.83 (d, 1H, J = 12.9 Hz, H-15b), 4.30 (d, 1H, J = 7.7 Hz, H-16), 3.33 (d, 1H, J = 6.9 Hz, H-17), 0.89 (s, 3H, H-18), 0.93 (s, 3H, H-19), 1.36 (s, 3H, H-21), 1.72 (m, 1H, H-23), 2.10 (m, 1H, H-24), 1.32 (s, 3H, H-26), 1.17 (s, 3H, H-27), 1.25 (s, 3H, H-28), 1.16 (s, 3H, H-29), 1.29 (s, 3H, H-30), 4.31 (d, 1H, J = 7.2 Hz, H- 1′), 3.35 (dd, 1H, J = 7.5 and 9.0 Hz, H-2′), 3.31 (dd, 1H, J = 7.5 and 9.5 Hz, H-3′), 3.36 (dd, 1H, J = 9.5 and 7.8 Hz, H-4′), 3.53 (m, 1H, H-5′), 4.04 (dd, 1H, J = 2.2 and 12.1 Hz, H-6a′), 3.86 (dd, 1H, J = 3.6 and 12.2 Hz, H-6b′). Negative FAB-MS *m/z* (rel. Int. %): 677 (30), 659 (19), 459 (10), 367 (25), 326 (32), 311 (50), 275 (100), 165 (60), 149 (85).

Tyrosinase Inhibitory Assay

Tyrosinase inhibition assays were performed in a 96-well microplate format using SpectraMax 340 microplate reader (Molecular Devices, CA, USA) according to the method developed earlier by Hearing (1987). First the compounds were screened for the *O*-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the active inhibitors from the preliminary screening were subjected to IC_{50} studies. Briefly, all the compounds were dissolved in DMSO giving a solution of 2.5%. Thirty units mushroom tyrosinase (28 nmol) were first preincubated with the compounds in 50 nmol Na-phosphate buffer (pH 6.8) for 10 min at 25°C. Then the L-DOPA (0.5 mmol) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37°C) of the formation of the DOPAchrome for 10 min. The percent inhibition of the enzyme and median inhibitory concentration (IC_{50}) of the active compounds were calculated using a program developed by Java and Macro with Excel 2000 (Microsoft Corp., USA) for this purpose:

$$\text{Percent inhibition} = [ABS_{Blank} - ABS_{Sample}/ ABS_{Blank}] \cdot 100$$

Here the ABS_{Blank} and ABS_{Sample} are the absorbances for the blank and samples, respectively. All the studies were done at least in triplicate and the results represent the mean SEM (standard error of the mean). All the regents, enzyme, substrate and reference compounds were purchased from Sigma Chem Co., MO, USA.

Results and Discussion

Isolation and identification of the compounds from C. colocynthis

A phytochemical investigation of the EtOAc and $CHCl_3$ fruit extracts of *C. colocynthis* was conducted in this study. A new fatty glycoside acid citrulloside (**1**), cucurbitacin E (**2**), 2-O-*β*-D-glucopyranosyl cucurbitacin E (**3**), 2-O-*β*-D-glucopyranosyl cucurbitacin I (**4**), 2-O-*β*-D-glucopyranosyl cucurbitacin L

(**5**) (Seger et al. 2005), *p*-hydroxybenzaldehyde, *p*-methoxymethyl phenol, *p*-hydroxybenzoic acid, *p*-hydroxybenzyl alcohol, and *β*-stigmasterol have been isolated from this plant.

Citrulloside (**1**) was isolated as a non-cristalline solid and its molecular formula $C_{40}H_{74}O_{10}$ was determined by positive-ion FABHRMS which exhibited the molecular ion $[M + H]^+$ at *m/z* 715.5418 (calc. 715.4838). The fragment ion at *m/z* 551 indicated the presence of one hexose moiety. The presence of one sugar in citrulloside **1** was also deduced from the negative-ion FABMS. The IR spectrum showed absorptions at 3373, 2919, 1725 and 1642 cm^{-1}, indicating the presence of hydroxyl groups, aliphatic groups, carboxylic acid and double bond respectively.

The UV spectra of **1** exhibited absorption at 204 nm, which confirmed the presence of a carboxylic acid. A very strong signal at δ 1.28 (40H, brs) in the ^1H NMR spectrum, corresponding to methylene groups revealed that **1** must be derived from a long-chain fatty acid precursor. The ^1H NMR spectrum (500 MHz, CD_3OD) of **1**, showed in addition to other signals, one anomeric proton at δ 4.25 (d, J = 7.7 Hz, H-1′), suggesting the presence of one sugar, *β*-D-glucopyranose unit. In the ^{13}C NMR spectrum (125 MHz, CD_3OD), the anomeric carbon atom appeared at δ 104.7. The anomeric proton at δ 4.25 showed a coupling constant of 7.7 Hz, indicating the presence of a *β*-D-glucosidic linkage (Agrawal 1992). The position of the the sugar residue was further deduced by 1-D-TOSCY, HMBC experiments and by acid hydrolysis. Acid hydrolysis afforded an aglycone and D-glucose, which were identified by comparative TLC with standard sugars using the solvent system BuOH/EtOAc/2-propanol/HOAc/H_2O (7:20:12:7:6). The detailed analysis of the ^1H NMR spectrum showed the presence of a fatty acid glucoside parent skeleton (Messanga et al. 2001). The cross-peak due to long range correlations between C-1 (δ 69.7) of the aglycone and H-1′ *β*-glucose (δ 4.25) and between C-1′ (δ 104.7) and H-1a,1b (δ 4.09, 3.70), established the glucosidic bond between C-1′ and C-1. The position of the C-11 oxymethine group was esthablised from the HMBC spectrum, in which the C-11 at δ 73.8 showed $^2J_{CH}$ correlation with H-10a (δ 1.69) and H-12a (δ 1.55), while the C-3 oxymethine proton at δ 4.12 showed $^3J_{CH}$ correlation with C-1 (δ 69.7) and C-4 (δ 131.9). Long range cross-peak correlations were also depicted between H-6 at δ 2.06 and C-5 (δ 134.3) and C-7 (δ 131.1) suggesting a linkage between C-5 and C-6. The location of the carboxyl group at C-2 was deduced from the HMBC interactions in which the carboxyl carbon at δ 177.4 showed an interaction with H-2 (δ 3.98). Finally, the cross-correlation peaks noticed between C-8 (δ 130.6) and H-9 (δ 1.96) and H-10a (δ 1.69) enabled the location of the double bond at C-7. The NOESY experiment confirmed the nature of the sugar moiety. The *trans* configuration of the double bond at C-4 was deduced from the large coupling constant 15.37 Hz found between H-4 and H-5, while the double bond at C-7 showed a *cis*-configuration (J = 7.3 Hz) because of the intense cross-peak noticed between H-8 and H-10a,10b. The relative stereochemistry of protons at C-2, C-3 and C-11 were esthablished from the interactions between H-2 / H-3, H-3 / H-5 and H-11 / H-10a in the NOESY experiment. The structure of **1** was confirmed by the analysis of the ion peaks in the mass spectrum. The linear aliphatic chain at the 12-position was assigned 21 carbon atoms because of the ion at *m/z* 296 derived from the fission of the C-12 / C-13 bond. The loss of a glucopyranosyl unit by **1** led to the ion *m/z* 534 and was followed by the elimination of a molecule of water to afford the ion *m/z* 516. The ^1H and ^{13}C NMR chemical shift assignments of compound **1** are based on the ^1H-^1H COSY, HMQC and HMBC spectra. The structure was therefore assigned as (4*E*,7*Z*)-2-carboxy-1-O-*β*-D-glucopyranosyl-3,11-dihydroxytritriaconta-4,7-diene.

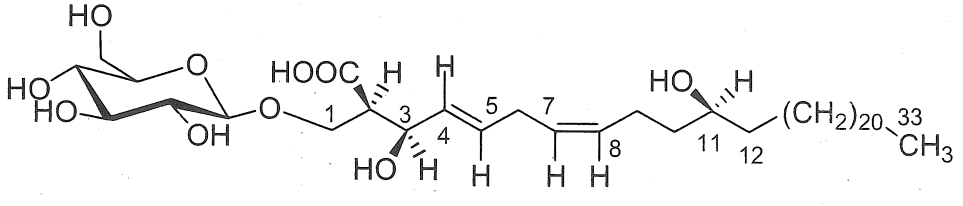

Figure 1. Structure of compound (**1**) isolated from *C. colocynthis*

4*E*,7*Z*-2-carboxy-1-O-*β*-D-glucopyranosyl-3,11-dihydroxytritriaconta-3,8-diene (**1**)

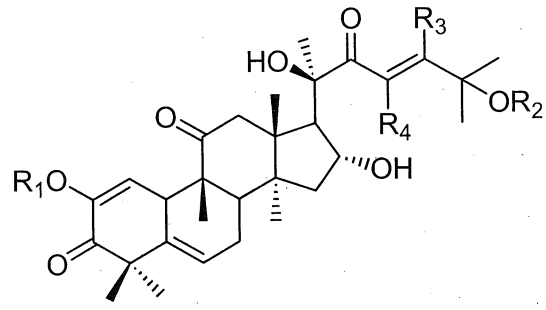

Figure 2. Structures of other compounds isolated from *C. colocynthis*

R^1	R^2	R^3	R^4	**Compounds**
H	Ac	H	H	cucurbitacin E (**2**)
D-glucose	Ac	H	H	2-O-*β*-D-glucopyranosyl cucurbitacin E (**3**)
D-glucose	H	H	H	2-O-*β*-D-glucopyranosyl cucurbitacin I (**4**)
D-glucose	H	2H	2H	2-O-*β*-D-glucopyranosyl cucurbitacin L (**5**)

Table 1. ^{13}C and ^{1}H NMR Assignments of compound 1 in CD_3OD[a]

Position	**δ (C)**	**δ (H)**
1	69.7 (CH_2)	4.09 (1H, dd, J 5.5, 10.3 Hz, H-1a) 3.70 (1H, dd, J 3.5, 10.3 Hz, H-1b)
2	54.6 (CH)	3.98 (1H, ddd, J 3.5, 5.5, 9.3 Hz)
3	72.9 (CH)	4.12 (1H, dd, J 7.4, 9.3 Hz)
4	131.9 (CH)	5.46 (1H, dd, J 7.4, 15.2 Hz)
5	134.3 (CH)	5.72 (1H, ddd, J 7.3, 9.3, 15.2 Hz)
6	33.6 (CH_2)	2.06 (2H, m)
7	131.1 (CH)	5.42 (1H, t, J 7.3 Hz)
8	130.6 (CH)	5.38 (1H, t, J 7.3 Hz)
9	33.3 (CH_2)	1.96 (2H, m)
10	35.7 (CH)	1.69 (1H, m, H-10a) 1.31 (1H, m, H-10b)
11	73.8 (CH)	3.96 (1H, m)
12	33.0 (CH_2)	1.55 (1H, m, H-12a) 1.40 (1H, m, H-12b)
13-32	23.7–30.7 ($(CH_2)_{20}$)	1.28 (40H, m, $(CH_2)_{20}$)
33	14.4 (CH)	0.89 (3H, t, 7 Hz)
1′	104.7 (CH)	4.25 (1H, d, J 7.7 Hz)
2′	77.9 (CH)	3.17 (1H, dd, J 7.7, 9.5 Hz)
3′	74.9 (CH)	3.34 (1H, dd, J 9.5, 9.5 Hz)
4′	71.5 (CH)	3.26 (1H, dd, J 9.5, 9.5 Hz)
5′	73.1 (CH)	3.67 (1H, m)
6′	62.6 (CH)	3.85 (1H, dd, J 2.0, 12 Hz, H-6′a) 3.64 (1H, dd, J 4.0, 12 Hz, H-6′b)
-	177.1 (COOH)	-

[a] ^{1}H NMR and ^{13}C NMR recorded at 500 and 125 MHz respectively

Inhibition of tyrosinase activity

Tyrosinase contains a strongly coupled binuclear copper active site and functions both as a monophenolase (monophenol + O_2 → *o*-diphenol + H_2O) and as an *o*-diphenolase (2*o*-diphenol + O_2 → 2*o*-quinone + 2 H_2O) (Kubo and Kinst-Hori 1998). A number of tyrosinase inhibitors have been reported but mostly as diphenolase inhibitors. Although monophenols such as L-tyrosine are important substrates, our knowledge about monophenolase inhibitors are still limited (Kubo and Kinst-Hori, 1998). In these experiments, the inhibitory effect of tyrosinase (E.C. 1.14.18.1) by compounds **1-5** was examined at the enzyme activity stage of the oxidation of L-DOPA to DOPAquinone. Their IC_{50} values are shown in the

Table 2 along with the well-known standard inhibitors of tyrosinase (kojic acid and L-mimosine). The results demonstrated that these compounds display a significant inhibition against the enzyme tyrosinase. Based on IC_{50} values, cucurbitacin E (**2**) (IC_{50}= 1.99 ∝mol) tended to be more active than the positive controls kojic acid (IC_{50}= 16.67 ∝mol) and L-mimosine (IC_{50}= 3.68 ∝mol). The substitution of the hydroxyl group at C-2 on the cucurbitacin skeleton by a glycosyl group significantly decreased the inhibition potency. Thus 2-O-β-D-glucopyranosyl cucurbitacin E (**3**), 2-O-β-D-glucopyranosyl cucurbitacin I (**4**) and, 2-O-β-D-glucopyranosyl cucurbitacin L (**5**) presented an IC_{50} of 35.50, 30.55 and 31.50 ∝mol respectively. Acetylation of the hydroxyl group at C-25 decreased the inhibitory effect: hence, 2-O-β-D-glucopyranosyl cucurbitacin E (**3**) showed a lower IC_{50} value (IC_{50} = 35.55 ∝mol) as compared with 2-O- β -D-glucopyranosyl cucurbitacin I (**4**) (IC_{50} = 30.55 ∝mol). The reduction of the double bond at C-23 of compound **4** to afford 2-O- β -D-glucopyranosyl cucurbitacin L (**5**) decreased the activity (IC_{50}= 31.50 ∝mol). The inhibitory effect of cucurbitacin E was explained by the structural similarirty between this compound with L-DOPA, the natural ligand, forming a similar skeleton and thus possibly acting as a competitive substrate to L-DOPA. The new compound **1**, a fatty acid glucoside, was inactive against tyrosinase.

From this study it is observed that, compounds isolated from this plant offer good candidates for the treatment of hyperpigmentation associated with the high production of melanocytes. Hyperpigmentation is associated with increased plasma melanocytes-stimulating hormone activity in people with insufficient production of glucocorticoids (Addison's disease). Recently, a number of inhibitors from natural products have been used in cosmetics (Lida et al. 1995). Further *in vitro* as well as *in vivo* studies on the activities of these compounds on melanocytes and skin pigmentation would be carried out with cucurbitacin E being the best possible candidate, with the aim of providing a more effective dermatological remedy for the treatment of hyperpigmentation and for its eventual use in cosmetics.

Table 2. The IC_{50} values of the compounds as compared with the IC_{50} values of the standard inhibitors of the enzyme tyrosinase

Compounds	**IC_{50} ± SEM (in ∝mol)**
Citrulloside (**1**)	Not active
Cucurbitacin E (**2**)	1.99 ± 0.0239
2-O-β-D-glucopyranosyl cucurbitacin E (**3**)	35.55 ± 0.0103
2-O-β-D-glucopyranosyl cucurbitacin I (**4**)	30.55 ± 0.0205
2-O-β-D-glucopyranosyl cucurbitacin L (**5**)	31.50 ± 0.305
Kojic acid[a]	16.67 ± 0.5190
L-Mimosine[a]	3.68 ± 0.02234

SEM is the standard error of the mean[a, b] are the standard inhibitors (KA and LM) of the enzyme tyrosinase
IC_{50} = 16.67 ∝mol

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