

Alliuocide G, a new flavonoid with potent α -amylase inhibitory activity from *Allium cepa* L.

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Abstract

Chemical investigation of the ethyl acetate fraction of the dried outer scales of *Allium cepa* L. afforded one new flavonoid named alliuocide G, together with four known compounds. Their structures were unambiguously established on the bases of NMR spectroscopy (^1H , ^{13}C , DEPT, and HMBC) and mass spectrometry. The isolated compounds showed potent α -amylase inhibitory activities, in addition the new compound had antioxidant activity.

Keywords: *Allium cepa* L. alliuocide G, antioxidant and α -amylase inhibitory activities

Introduction

High dietary intakes of fruits and vegetables have been reported to correlate with a low risk of degenerative diseases from epidemiological evidence. The protective effects have been attributed partly to the various antioxidative compounds present in fruits and vegetables. The most abundant types of antioxidative compounds in human diet are flavonoids¹. Previous phytochemical studies of the *A. Cepa* L. (family: Liliaceae) (white and red onion) have resulted the isolation of flavonoids²⁻⁶, especially high levels of quercetin and its derivatives², anthocyanins^{7, 8}, thiosulfinates, sulfides, sulfoxides³ and peptides⁹. This study undertook the isolation and structure elucidation of one new flavonoid named alliuocide G, together with four known compounds. The isolated compounds were tested for their α -amylase inhibitory activities, in addition the new compound was evaluated for its antioxidant activity using DPPH assay.

Results and Discussion

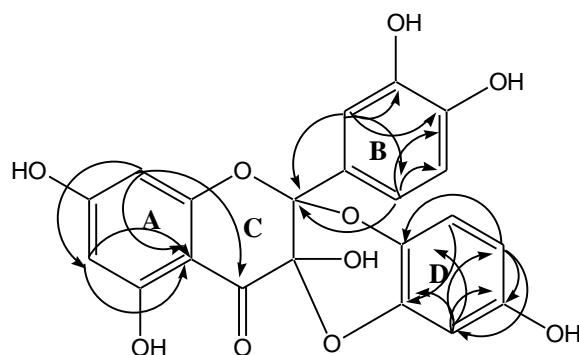
The methanolic extract of the air-dried brownish scales of onions was concentrated to dryness and successively partitioned between *n*-hexane, ethyl acetate, *n*-butanol and water. The ethyl

acetate fraction afforded one new flavonoid named alliuocide G (**2**) and four known compounds (**1**, **3-5**).

Compound **2** was obtained as brownish amorphous powder (18.6 mg). It had a molecular weight of 426 as derived from the ESI-MS measurement which in conjunction with ^1H , ^{13}C and DEPT NMR spectral data suggested the molecular formula $\text{C}_{21}\text{H}_{14}\text{O}_{10}$. The UV spectrum showed absorbance at λ_{max} (MeOH) 290 nm indicated the lack of conjugation between C-2 and C-3 of the quercetin moiety¹. IR spectrum showed absorption bands at γ_{max} (KBr) cm^{-1} ; 3455 (OH), 1658 (C=O) and 1556 (C=C). The ^1H NMR spectrum showed the presence of two protons at δ_{H} 5.94 (brs, H-6/8), one *ortho* coupled at δ_{H} 6.67 (d, $J = 8.5$ Hz, H-5'), one *ortho* and *meta* coupled at δ_{H} 6.90 (dd, $J = 8.5, 1.8$ Hz, H-6') and one *meta* downfield coupled proton at δ_{H} 7.10 (d, $J = 1.8$ Hz, H-2') indicated the presence of quercetin moiety. In addition, three proton signals at δ_{H} 7.15 (d, $J = 8.5$ Hz, H-5''), 7.46 (d, $J = 1.9$ Hz, H-2'') and 7.58 (dd, $J = 8.5, 1.9$ Hz, H-6'') were observed, attributed to a *tri*-substituted benzene moiety. The ^{13}C spectrum showed resonances for twenty one carbon signals, one downfield quaternary signal at δ_{C} 187.1 (C-4), two characteristic quaternary carbons at δ_{C} 100.0 (C-2) and 90.8 (C-3). These data indicated that compound **2** consisting of quercetin in addition to a *tri*-substituted benzene moiety, in which oxidative coupling of both conjugated olefinic linkage (C-2 and C-3) of the quercetin C-ring and the *ortho*-dihydroxy group (C-3'' and C-4') of 1,3,4-trihydroxy benzene ring. The structure assignment was supported by HMBC spectral analysis of cross peaks: the proton signal H-2'' gave cross peaks with carbon signals resonating at δ_{C} 124.4 (C-6''), 140.3 (C-3''), 144.7 (C-4') and 166.1 (C-1'), H-5'' with C-6'' (δ_{C} 124.4), C-3'' (δ_{C} 140.3), C-4'' (δ_{C} 144.7), H-6'' with C-2'' (δ_{C} 117.9), C-5'' (δ_{C} 118.4), C-4'' (δ_{C} 144.7), and C-1'' (δ_{C} 166.1). Furthermore, H-6 showed HMBC correlations with C-5 (δ_{C} 163.1), C-7 (δ_{C} 166.1), C-8 (δ_{C} 96.3) and H-8 with C-4 (δ_{C} 187.1), C-6 (δ_{C} 97.3), C-9 (δ_{C} 159.1) and C-10 (δ_{C} 100.2). In addition, the connectivity of ring-D to ring-C at C-2 and C-3 of quercetin moiety was confirmed by the upfield shifts of C-2 (δ_{C} 100.2) and C-3 (δ_{C} 90.8). To the best of our knowledge compound **2** is a new natural product and named alliuocide G. The known compounds were identified by analysis of the spectroscopic data (NMR and MS) and comparison of their data with those reported in the literature to be: 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3 (2*H*)-benzofuranone (**1**)¹, 1,3,11 α -trihydroxy-9-(3,5,7-trihydroxy-4*H*-1-benzopyran-7-on-2-yl)-5 α -(3,4-dihydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one (quercetin dimer) (**3**)^{1,5}, luteolin-7-*O*- β -D-glucopyranoside (**4**)^{10,11} and [1,3,11 α -trihydroxy-9-(3,5,7-trihydroxy-4*H*-1-benzopyran-7-on-2-yl)-5 α -(3,4-dihydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxana-phthacene-12-one]-4'-*O*- β -D-glucopyranoside (4'-*O*- β -D-glucopyranoside of quercetin dimer) (**5**)^{1,5}. This is the first report of compound (**4**) in the plant, while compounds (**1**, **3** and **5**) were previously isolated from the same plant^{1,5}.

Table 1. ^1H and ^{13}C NMR data of compound **2** (DMSO- d_6 , 500, 125 MHz)

Pos.	δ_{H} (J Hz, m)	δ_{C} (m)	HMBC
2		100.0 s	
3		90.8 s	
4		187.1 s	
5		163.1 s	
6	5.94, 1H, brs	97.3 d	5, 7, 8, 10
7		166.6 s	
8	5.94, 1H, brs	96.3 d	4, 6, 9, 10
9		159.1 s	
10		100.2 s	
1'		124.2 s	
2'	7.10, 1H, d, $J = 1.8$ Hz	115.7 d	2, 3', 4', 6'
3'		144.4 s	
4'		147.0 s	
5'	6.67, 1H, d, $J = 8.5$ Hz	114.8 d	1', 3', 4'
6'	6.90, 1H, dd, $J = 8.5, 1.8$ Hz	119.3 d	2, 5', 4'
1''		166.6 s	
2''	7.46, 1H, d, $J = 1.9$ Hz	117.9 d	1'', 3'', 4'', 6''
3''		140.3 s	
4''		144.7 s	
5''	7.15, 1H, d, $J = 8.5$ Hz	118.4 d	3'', 4'', 6''
6''	7.58, 1H, dd, $J = 8.5, 1.9$ Hz	124.4 d	1'', 2'', 4'', 5''
OH	10.85, 9.18, 8.89, each 1H, s		

**Figure 1.** Some important HMBC correlations of compound **2**.

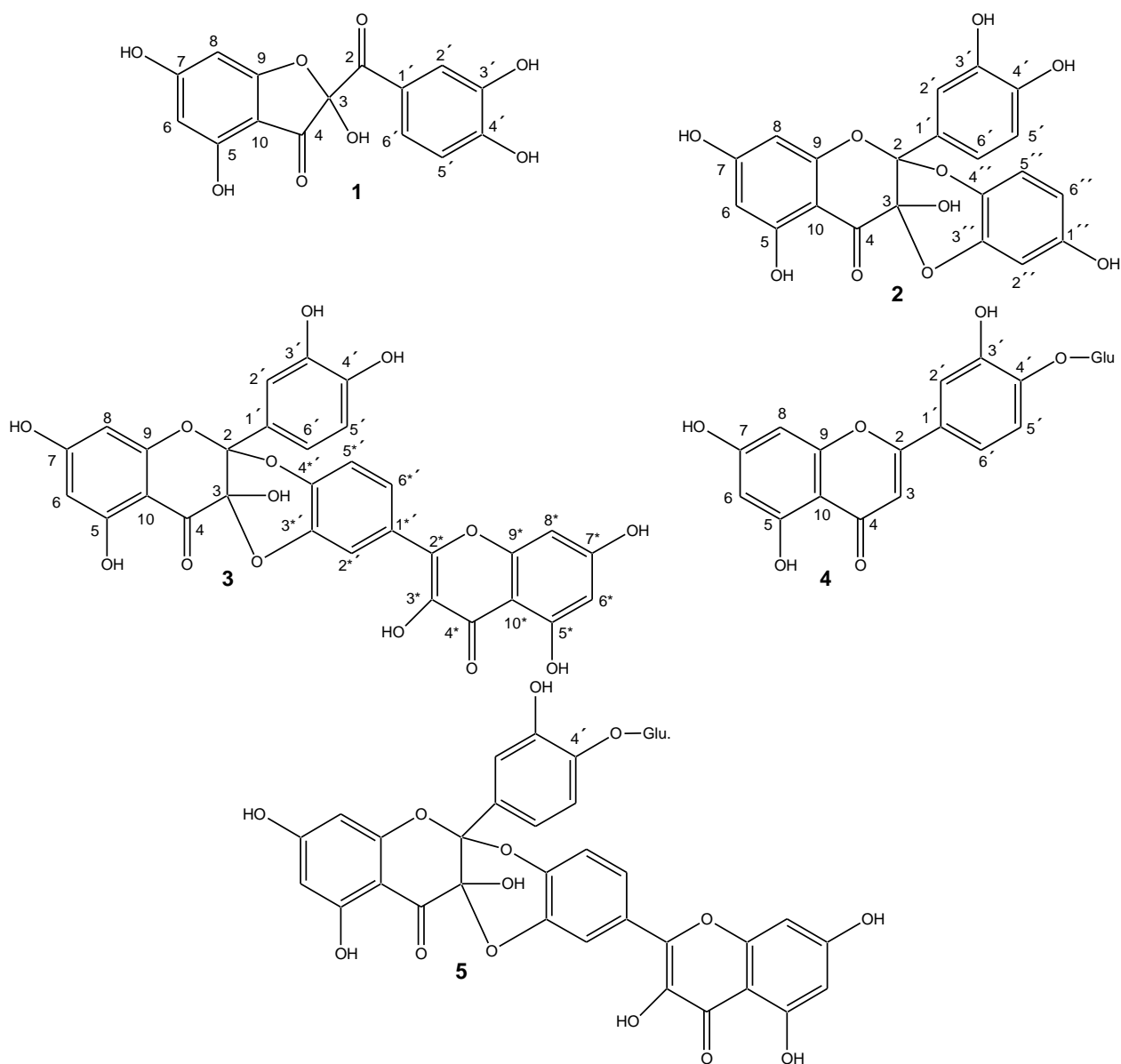


Figure 2. Chemical structures of isolated compounds 1-5.

Experimental Section

General Procedures. Melting points were carried out in Electrothermal 9100 Digital Melting Point (England, Ltd). The UV spectra were carried out in methanol (Merck) using a Perkin-Elmer Lambda 25 UV/VIS spectrophotometer. IR was measured on Shimadzu Infrared-400 spectrophotometer (Japan). Optical rotation was recorded on a Perkin-Elmer Model 341 LC Polarimeter. α -Amylase inhibitory activities were measured in Tecan Genios microplate reader

(GmbH, Germany). 1D and 2D NMR experiments were performed on Bruker Unity 500, 125 MHz spectrometer. ESI-MS spectra were obtained with a Thermofinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. Column chromatographic separation were performed on silica gel 60 (0.04-0.063 mm), RP-18 (0.04-0.063 mm Merck), and Sephadex LH-20 (0.25-0.1 mm Merck). TLC analyses were carried out on aluminum sheets precoated with silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany). The solvent systems used for TLC analyses were CH₂Cl₂-MeOH (95:5, solvent system I), CH₂Cl₂-MeOH (9:1, solvent system II) and CH₂Cl₂-MeOH (85:15, solvent system III). All solvents were distilled prior to use. Spectral grade solvents were utilized for chromatographic analysis.

Extraction and isolation

White onions were purchased at the Faculty of Agriculture, Al-Azhar University, Assiut, Egypt. The air-dried brownish scales of onions (400 g) was extracted several times with methanol (2 L × 5) and evaporated to yield 36 g. The latter was suspended in distilled water (100 ml) then partitioned between *n*-hexane (500 mL × 4), ethyl acetate (500 mL × 4) and *n*-butanol (500 mL × 3), successively. Each fraction was concentrated under reduced pressure to give *n*-hexane fraction (4.6 g), ethyl acetate fraction (8.6 g), *n*-butanol fraction (10.3 g) and aqueous (12.2 g) residue. The ethyl acetate fraction (8.6 g) was subjected to vacuum liquid chromatography (VLC) using CHCl₃-MeOH gradients to afford 5 fractions. Fraction II (0.96 g) was chromatographed over silica gel column using CHCl₃-MeOH gradients to get compound **1** (32 mg). Fraction III (2.3 g) was chromatographed on Sephadex LH-20 eluted with MeOH to yield subfractions A-C. Subfraction A (0.38 g) was subjected to silica gel column using CHCl₃-MeOH gradients to afford compound **3** (26 mg). Subfraction C (0.71 g) was subjected to RP-18 column using MeOH-H₂O gradient to give compound **2** (18 mg). Fraction V (3.7 g) was chromatographed over silica gel column using CHCl₃-MeOH gradients to obtain compound **4** (13 mg), and **5** (44 mg). Further purification of compounds **4** and **5** were accomplished by RP-18 column eluted with MeOH-H₂O gradients.

2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxy-3 (2H)-benzofuranone (1).¹ Brown amorphous solid; R_f = 0.53 (solvent system I); m.p. 213-214 °C, UV λ_{max} (MeOH): 292 nm. (-) ESI-MS *m/z* (rel. int.%): 317.2 [M-H]⁻ (100). ¹H NMR data (DMSO-*d*₆, 500 MHz): δ_H 5.89 (1H, brs, H-6), 5.94 (1H, brs, H-8), 6.79 (1H, d, *J* = 8.3 Hz, H-5'), 7.54 (1H, dd, *J* = 8.3, 1.9 Hz, H-6'), 7.55 (1H, d, *J* = 1.9 Hz, H-2'), 9.46 and 8.69 (each 1H, s, OH-groups).

1,3,9,11α-Tetrahydroxy-5α-(3,4-dihydroxyphenyl)-5,11-dihydro-5,6,11-trioxa naphthacene-12-one (Alliuocide G) (2). Brownish amorphous solid; R_f = 0.71 (solvent system II); [α]_D + 71.4° (*c* 0.5, MeOH). m.p. 292-293 °C, UV λ_{max} (MeOH): 290 nm. (+) ESI-MS *m/z* (rel. int.%): 427.2 [M+H]⁺ (100), (-) ESI-MS *m/z* (rel. int.%): 425.1 [M-H]⁻ (86). IR γ_{max} (KBr) cm⁻¹: 3455, 2986, 1658, 1556, 1057, 583. ¹H NMR data (DMSO-*d*₆, 500 MHz): δ_H 5.94 (2H, brs, H-6/8), 6.67 (1H, d, *J* = 8.5 Hz, H-5'), 6.90 (1H, dd, *J* = 8.5, 1.8 Hz, H-6'), 7.10 (1H, d, *J* = 1.8 Hz, H-2'), 7.15 (1H, d, *J* = 8.5 Hz, H-5''), 7.46 (1H, d, *J* = 1.9 Hz, H-2''), 7.58 (1H, dd, *J* = 8.5, 1.9

Hz, H-6''), 8.89, 9.18 and 10.85 (each 1H, s, OH-groups). ¹³C NMR (DMSO-*d*₆, 125 MHz): δ_C 90.8 (C-3, s), 96.3 (C-8, d), 97.3 (C-6, d), 100.0 (C-2, s), 100.2 (C-10, s), 114.8 (C-5', d), 115.7 (C-2', d), 117.9 (C-2'', d), 118.4 (C-5'', d), 119.3 (C-6', d), 124.2 (C-1', s), 124.4 (C-6'', d), 140.3 (C-3'', s), 144.4 (C-3', s), 144.7 (C-4'', s), 147.0 (C-4', d), 159.1 (C-9, s), 163.1 (C-5, s), 166.6 (C-7, 1'', s), 187.1 (C-4, s).

1,3,11α-Trihydroxy-9-(3,5,7-trihydroxy-4H-1-benzopyran-7-on-2-yl)-5α-(3,4-dihydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one (Quercetin dimer) (3).^{1, 5}

Yellow amorphous solid; R_f = 0.64 (solvent system II); m.p. 267-268 °C, UV λ_{max} (MeOH): 254, 272, 300, and 370 nm. (-) ESI-MS *m/z* (rel. int.%): 601.1 [M-H]⁻ (100). ¹H NMR data (DMSO-*d*₆, 500 MHz): δ_H 5.97 (2H, brs, H-6/8), 6.20 (1H, d, *J* = 2.8 Hz, H-6*), 6.48 (1H, d, *J* = 2.7 Hz, H-8*), 6.71 (1H, d, *J* = 8.5 Hz, H-5'), 6.95 (1H, dd, *J* = 8.5, 2.2 Hz, H-6'), 7.17 (1H, d, *J* = 2.2 Hz, H-2'), 7.26 (1H, d, *J* = 8.5 Hz, H-5*''), 7.84 (1H, dd, *J* = 8.5, 2.2 Hz, H-6*''), 7.91 (1H, d, *J* = 2.2 Hz, H-2*''), 10.99, 9.21 and 8.97 (each 1H, s, OH-groups). ¹³C NMR (DMSO-*d*₆, 125 MHz): δ_C 91.4 (C-3, s), 93.7 (C-8*, d), 96.4 (C-8, d), 97.3 (C-6, d), 98.4 (C-6*, d), 99.8 (C-2, s), 100.2 (C-10, s), 103.3 (C-10*, s), 114.9 (C-5', d), 115.7 (C-2', d), 116.4 (C-5*''), d), 117.6 (C-2*''), d), 119.3 (C-6', d), 122.5 (C-6*''), d), 124.5 (C-1', s), 125.6 (C-1*''), s), 136.9 (C-3*, s), 140.5 (C-3*''), s), 142.5 (C-4*''), s), 144.8 (C-2*, s), 145.2 (C-3', s), 147.1 (C-4', s), 156.4 (C-9*, s), 159.2 (C-9, s), 160.8 (C-5*, s), 163.1 (C-5, s), 164.3 (C-7*, s), 168.1 (C-7, s), 176.2 (C-4*, s), 187.3 (C-4, s).

Luteolin-7-O-β-D-glucopyranoside (4).^{10, 11} Yellow amorphous solid; R_f = 0.86 (solvent system III); m.p. 244-245 °C, UV λ_{max} (MeOH): 266 and 344 nm. +NaOMe: 273, 300 sh, 389; +AlCl₃: 283, 298 sh, 329, 425; +AlCl₃/HCl: 281, 294 sh, 358, 384; +NaOAc: 270, 276, 367 sh, 394; +NaOAc/H₃BO₃: 270, 368. (+) ESI-MS *m/z* (rel. int.%): 449.1 [M+H]⁺ (100), 286.9 [M-162 (glucose)]⁺ (100), (-) ESI-MS *m/z* (rel. int.%): 447.3 [M-H]⁻ (100). ¹H NMR data (DMSO-*d*₆, 500 MHz): δ_H 4.94 (1H, d, *J* = 7.1 Hz, H-1''), 3.44-5.12 (m, other sugar protons), 6.51 (1H, brs, H-6), 6.56 (1H, brs, H-8), 6.98 (1H, s, H-3), 7.41 (1H, d, *J* = 8.8 Hz, H-5'), 7.91 (1H, dd, *J* = 8.8, 2.2 Hz, H-6'), 8.00 (1H, d, *J* = 2.2 Hz, H-2'), 12.86 (1H, s, 5-OH).

[1,3,11α-Trihydroxy-9-(3,5,7-trihydroxy-4H-1-benzopyran-7-on-2-yl)-5α-(3,4-dihydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one]-4'-O-β-D-glucopyranoside (4'-O-β-D-glucopyranoside of quercetin dimer) (5).^{1, 5}

Yellow amorphous solid; R_f = 0.76 (solvent system III); m.p. 301-302 °C, UV λ_{max} (MeOH): 254, 275, 303 and 376 nm. (+) ESI-MS *m/z* (rel. int.%): 765.3 [M+H]⁺ (100), 602.9 [M-162 (glucose)]⁺ (76), (-) ESI-MS *m/z* (rel. int.%): 763.2 [M-H]⁻ (100). ¹H NMR data (DMSO-*d*₆, 500 MHz): δ_H 4.92 (1H, d, *J* = 7.25 Hz, H-1''), 3.45-5.49 (m, other sugar protons), 6.20 (1H, d, *J* = 2.2 Hz, H-6), 6.37 (1H, d, *J* = 2.2 Hz, H-8), 6.40 (1H, d, *J* = 2.2 Hz, H-6*), 6.80 (1H, d, *J* = 2.2 Hz, H-8*), 7.20 (1H, d, *J* = 8.8 Hz, H-5'), 7.26 (1H, d, *J* = 8.6 Hz, H-5*'') 7.42 (1H, d, *J* = 2.2 Hz, H-2'), 7.52 (1H, m, H-6'), 7.65 (1H, m, H-6*''), 7.74 (1H, d, *J* = 2.2 Hz, H-2*''), 12.58, 12.37, 10.22, 9.69, and 8.79 (each 1H, s, OH-groups). ¹³C NMR (DMSO-*d*₆, 125 MHz): δ_C 60.5 (C-6'', t), 69.9 (C-4'', d), 73.2 (C-2'', d), 77.1 (C-5'', d), 77.6 (C-3'', d), 90.9 (C-3, s), 94.3 (C-8*, d), 96.9 (C-8, d), 98.2 (C-6, d), 99.1 (C-6*, d), 100.7 (C-2, s), 101.3 (C-10, s), 104.3 (C-10*, s), 104.0 (C-1'', d), 115.4 (C-5', d), 116.4 (C-2', 5*''), d), 117.1 (C-2*''), d), 120.9 (C-6', d), 124.3 (C-6*''), d), 129.9 (C-1', s), 125.6

(C-1^{*}, s), 138.1 (C-3^{*}, s), 141.7 (C-3^{*}, s), 142.9 (C-4^{*}, s), 145.9 (C-2^{*}, s), 146.2 (C-3['], s), 146.9 (C-4['], s), 158.2 (C-9^{*}, s), 160.4 (C-9, s), 162.8 (C-5^{*}, s), 165.3 (C-5, s), 165.4 (C-7^{*}, s), 168.9 (C-7, s), 177.4 (C-4^{*}, s), 188.6 (C-4, s).

α -Amylase inhibitory activity^{10, 12}

The method is based on assay of α -amylase by EnzCheck[®] Amylase Assay Kit (E-11954) was purchased from Molecular Probes (GmbH, Germany). The provided stock solution of DQ starch and α -amylase enzyme were diluted with the reaction buffer (pH 6.9) according to the reported protocol¹². To the microplate wells, 50 μ g/10 μ L of the tested compound in DMSO, 50 μ L of the diluted enzyme and 40 μ L of the reaction buffer were added and allowed to stand for 5 min at room temperature then 100 μ L of DQ starch was added. The fluorescence intensity of the digestion products from the DQ starch (with or without compounds) was measured using a kinetic assay program in the Tecan Genios microplate reader at λ_{max} 485 \pm 10 nm starting from zero min to 60 min at 10 min intervals. All determinations were performed in triplicate. The α -amylase inhibitory activity of each tested compound was measured in relation to acarbose (Ac) (Kohlpharmam GmbH, Germany) set as 100% α -amylase inhibitory activity. The percentage of α -amylase activity and α -amylase inhibition was calculated using the following equations:

$$\% \alpha\text{-amylase activity} = 100 \times \frac{(F_s^{60} - F_s^0)}{(F_c^{60} - F_c^0)}$$

where F_s^{60} : Fluorescence with the sample at 60 min, F_s^0 : Fluorescence with the sample at 0 min, F_c^{60} : Fluorescence of the control at 60 min, F_c^0 : Fluorescence of the control at 0 min.
 $\% \alpha$ -amylase inhibition = 100 – $\% \alpha$ -amylase activity.

Table 2. α -Amylase inhibitory activity of the isolated compounds

Compounds	Inhibition %
1	88.7
2	96.5
3	87.7
4	82.8
5	56.4

All isolated compounds were evaluated for α -amylase inhibitory activity as shown in table 2, new compound **2** showed potent activity compared with acarbose. Apparently, α -amylase inhibitory activity increased with the presence of hydroxyl groups at 3['], and 4[']-position of the B-ring¹³.

Free radical scavenging activity (DPPH assay)¹⁴

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and propyl gallate (PG) as reference sample were obtained from Sigma Chemical Co. (Germany). The method was previously described¹⁴.

Compound **2** was dissolved in HPLC MeOH to obtain a concentration of 20 μm /mL. Then was mixed with DPPH (118×10^{-5}) and allowed to stand for half an hour for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted. The antioxidant activity was calculated using the following equation;

$$\text{Antioxidant activity} = 100 \times \left(1 - \frac{\text{absorbance with compound}}{\text{absorbance of the blank}} \right)$$

Compound **2** have 66.4 % antioxidant activity according to published structure activity relationship of flavonoids¹⁵⁻¹⁸ compared with propyl gallate (a known synthetic antioxidant) set as 100 % antioxidant activity.

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