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Grandisin, 2-methoxy 6,7,2',6'-tetrahydroxy flavanone 6-*O*-glucoside, from *Cassia grandis* leaves - antioxidant and cytotoxic activities

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Chemical investigation of *Cassia grandis* leaves resulted in the isolation of the new 2-methoxy 6,7,2',6'-tetrahydroxy flavanone 6-*O*-β-glucoside together with the known flavonol glycosides, kaempferol-3-*O*-α-rhamnoside, and quercetin 3-*O*-α-rhamnoside. The structure assignments were based on conventional analytical methods and confirmed by HRFTESIMS, ^{1}H and ^{13}C NMR, COSY, HSQC and HMBC data. The total phenolic content of the extract was estimated by Folin-Ciocalteu's method. The antioxidant capacity was investigated using DPPH radical scavenging assay. The ethyl acetate and the *n*-butanol fractions showed poor cytotoxic activity only at high concentrations against the three different cancer cell lines, hepatocellular (HepG-2), breast (MCF-7), and prostate (PC3) by the neutral red uptake assay.

1. Introduction

Following our interest in the search of biologically active plant extracts and studying their constitutive phenolic metabolites (Ayoub et al. 2013; Hussein et al. 2013; Ayoub et al. 2009), we investigated the antioxidant and cytotoxic activities of *Cassia grandis* (Fabaceae) methanolic extract. Besides, we studied the major phenolic metabolites of that extract.

The genus *Cassia* comprises a group of flowering plants in the legume family, Fabaceae, and the subfamily Caesalpinioideae. The species are known commonly as cassias and are widely distributed throughout the world including Asia, East Africa, South Africa, America, Mexico, West Indies, and Brazil. Some important species are *Cassia fistula, C. grandis, C. hirsutica*, *C. sieberiena*, *C. alata*, *C. tora*, *C. occidentalis, C. auriculata*, and *C. nigricans* (Ayo et al. 2007). It was recognized that *C. grandis* is not well explored so far, except for one report on its antidiabetic potential. The aqueous and ethanolic extracts showed that they significantly lowered blood glucose levels in normal rats and alloxan induced diabetic rats to normal in the glucose tolerance test (Lodha et al. 2010). Hence, the present study has been undertaken to investigate the constitutive phenolics of the aqueous methanol leaf extract of *C. grandis* in association with its antioxidant capacity and cytotoxic activity as well. Thus, we were able to isolate and characterize a novel flavanone glucoside (**3**) and the known flavonoids, kaempferol -3*O*-rhamnoside (**1**) and quercetin 3-*O*-rhamnoside (**2**). It should be noted that the antioxidant and cytotoxic investigation of the extract of *C. grandis* were not reported previously as far as the available current literature is concerned.

2. Investigations, results and discussion

2.1. Total phenolic content in C. grandis leaves

Phenolic compounds have been recognized to possess strong antioxidant properties. The antioxidant activity of phenolic compounds is mainly due to their redox properties which allow them to act as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers (Rice-Evans et al. 1997). The total phenolic content in *C. grandis* aqueous methanolic extract was found to be 0.2±0.033 mg of GAEs/g of extract.

2.2. Isolation and structure elucidation

2.2.1. Known compounds

Following fractionation of the aqueous methanolic extract of *C. grandis* leaves, and investigation of the *n*-butanol and ethyl acetate fraction, two major phenolic compounds were found predominant in the two fractions. Chromatographic behavior, UV spectral, ESI-MS (negative mode), ^{1}H and ^{13}C NMR data were consistent with those previously reported for kaempferol 3-*O*-α-L-rhamnopyranoside (**1**), and quercetin 3-O-α-L-rhamnopyranoside (**2**) (Hussein et al. 2013).

2.2.2. Identification of the new natural product 2-methoxy 6,7,2',6'-tetrahydroxy flavanone 6-O-glucoside

Compound **3** was isolated as a pale yellow amorphous powder which exhibited chromatographic (dark purple spot under short UV light which turned blue green when sprayed with $FeCl_3$) and UV spectral characteristics: λ_{max}280 nm in MeOH which indicates the presence of a benzonoid chromophore and the lack of conjugation reminiscent with flavonones. The IR spectrum of **3** showed strong absorption at 3423, 2918, 2850, 1718, 1628 cm^{-1} consistent with the presence of phenolic hydroxyl, methoxyl, carbonyl and benzenoid C=C groups, respectively. HRESIFTMS (positive mode) showed *m/ z* = 485.1058 (M+ Na)$^{+}$ corresponding to a molecular formula of

$C_{22}H_{22}O_{11}Na$, thus indicating a molecular formula of $C_{22}H_{24}O_{12}$ and a molecular mass of 480 mU. The ^{1}H-NMR spectrum of **3** showed eight signals and the correlations recognized in the HSQC spectrum together that in the ^{1}H-^{1}H COSY spectrum allowed the definition of these protons as follows: two para aromatic methines at δ ppm 7.36 and 7.59 (each broad s, $\Delta\nu_{1/2}$=4 Hz); three upfield sp^{2} methines protons, assignale to a 3, 4, 5 tri-substitued benzene

protons at δ ppm 6.16 (2 H, *d*, *J*=8 Hz,) 7.16 (1H, *t*, *J*=8 Hz), a sp^3methine proton at δ ppm4.55, along with a methoxy group attached to a sp^3 carbon at δ ppm 3.66 (s,3H) and an anomeric sugar proton at δ ppm 4.81 (*d*, *J*=6.4 Hz). The pattern of proton signals in the aromatic region of the ^{1}H NMR spectrum between δ 7.59 and 6.16 when incorporated in the above given analytical data suggests a flavanone moiety, bearing di-para hydroxyl group at the A-ring , di-meta hydroxyl groups at the B-ring and a substituted C-2 with methoxyl group at the C ring. This view was supported by ^{13}C, COSY, HSQC and HMBC NMR data (Table).

Table: 1D and 2D NMR data of compound 3, (δ ppm, *J* in Hz, in DMSO-d6)

Position	δ H (*J* in Hz)	^{1}H-^{1}H COSY	δ C	APT	HMBC
2	-		127.5	Quaternary	
3	4.55 (*s*)		62.70	Methylene	C-2, C-4, C-10
4	-		199.86	Quaternary	
5	5.59 (*br. s.*)	H-5, H-8	120.72	Methine	C-3, C-6, C-7
6	-		144.18	Quaternary	C-9
7	-		153.64	Quaternary	
8	7.36 (*br. s.*)	H-8, H-5	118.11	Methine	
9	-		162.0	Quaternary	C-3, C-5, C-10
10	-		134.21	Quaternary	
1'	-		111.79	Quaternary	
2'	-		165.97	Quaternary	
3'	6.16(*d*,8Hz)	H-3', H-4'	107.0	Methine	
4'	7.16(*t*, 8Hz)	H-4', H-3'	136.92	Methine	C-2', C-1'
5'	6.16(*d*,8Hz)	H-5'	107.0	Methine	C-2', C-1', C-5'
6'	-	H-5', H-4'	165.97	Quaternary	C-2', C-1'
OCH_3	3.66 (*s*)		52.71		C-3, C-4
1''	4.81(*d*,4Hz)		101.64		
2''			73.78		
3''			76.98		
4''			69.91		
5''			77.31		
6''			62.7		

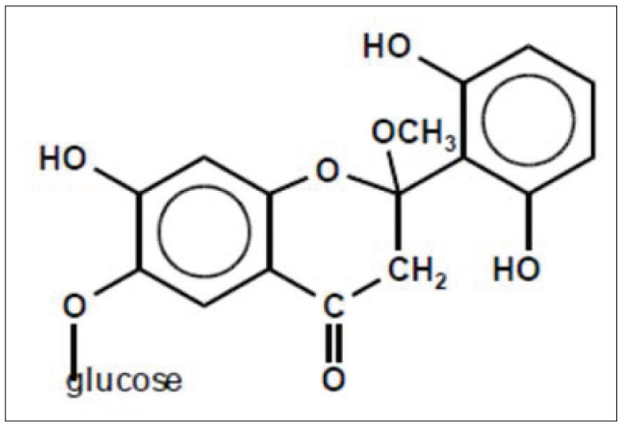

Fig. 1: Compound 3

Among the twenty two carbon signals recognized in the ^{13}C spectrum of **3**, fifteen carbons possessed chemical shift values consistent with 6,7,2',6'tetrahydroxylated flavanone in which the C-2 sp^3 carbon of ring C at δ127.5 ppm is substituted by a methoxy group (δ 52.71 ppm). The data obtained from the NMR experiments for the remaining six carbon signals were found to be best interpreted in terms of a β- glucoside moiety which is connected to C-6 of the flavanone aglycone, thus forming the molecule of **3**. Among the ^3J correlations recognized in the HMBC spectrum, one was found correlating the methylene

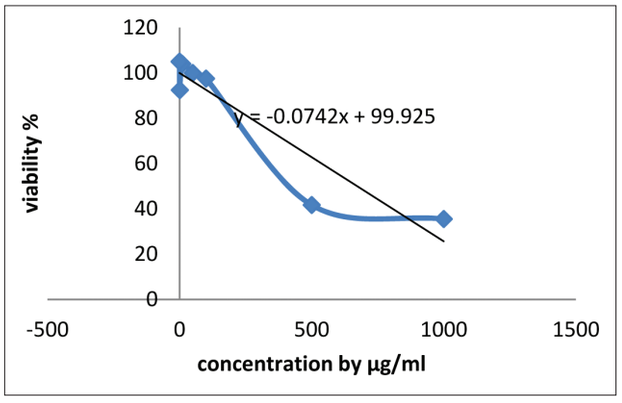

Fig. 2: Cytotoxic activity of the butanol fraction against PC3 cell line

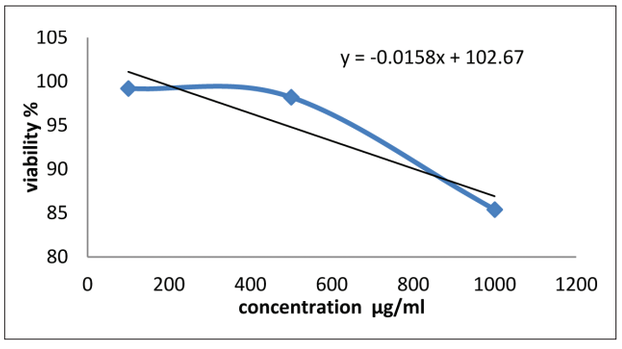

Fig. 3: Cytotoxic activity of the butanol fraction against HepG-2 cell line

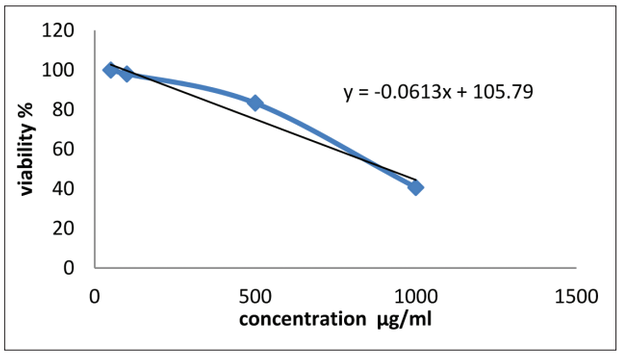

Fig. 4: Cytotoxic activity of the butanol fraction against MCF-7 cell line

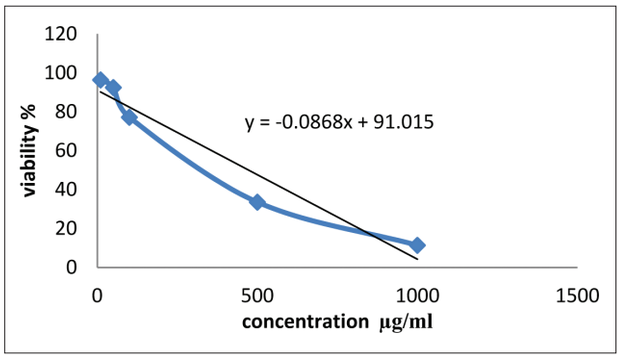

Fig. 5: Cytotoxic activity of the butanol fraction against MCF-7 cell line

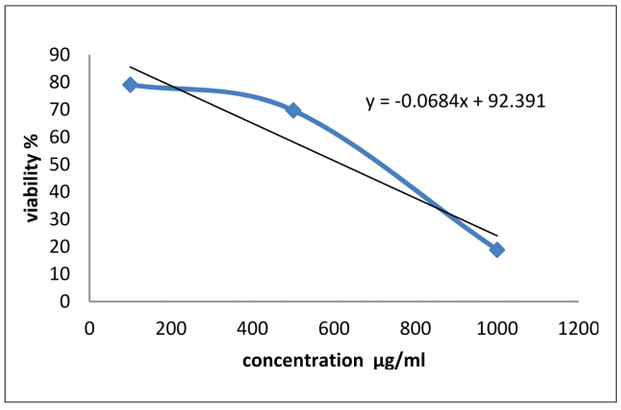

Fig. 6: Cytotoxic activity of the ethyl acetate fraction against HepG-2 cell line

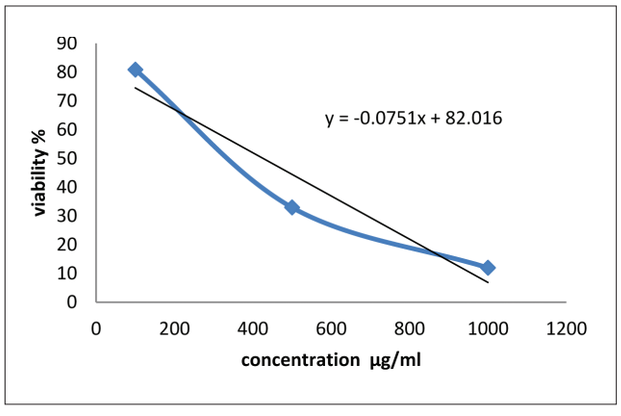

Fig. 7: Cytotoxic activity of the ethyl acetate fraction against MCF-7 cell line

H-3 proton signal at δ 4.55 to the benzenoid C-10 carbon signal at δ 134.21and another one correlated the benzenoid proton H-5 to C-7 at δ153.64. The methoxyl protons at δ 3.66 ppm was correlated in this spectrum via a ^3J coupling to C-2 of the pyranose ring. Detailed direct correlations observable in the HMBC spectrum are given in Table).

These and the above given data finally confirmed the structure of compound **3** to 2-methoxy 6,7,2',6'-tetrahyddroxylated flavanone 6-*O*-β glucoside or grandisin (Fig. 1), a new flavanone β-*O*-glucoside, which has not been previously reported to occur in nature.

2.3. DPPH radical scavenging activity

For ethyl acetate fraction, IC_{50}= 34.29 µg/ m; for *n*-butanol fraction, IC_{50} = 37.08 µg/ml; for the total methanolic extract, IC_{50} = 60.67 µg/ ml.

2.4. In vitro neutral red cytotoxicity assay

The cytotoxic activity of the ethyl acetate and butanol fractions was inspected against three human tumor cell lines. The two fractions exhibited poor cytotoxic activity on the cell lines tested and only at high concentrations. The IC_{50} values of the butanol fraction were 672.5 µg/ml (Fig. 2), 3606.6 µg/ml (Fig. 3) and 913 µg/ml (Fig. 4) for PC3, HepG-2 and MCF-7 cell lines respectively. While the ethyl acetate fraction revealed IC_{50} values of 472.35 µg/ml (Fig. 5), 623.38 µg/ml (Fig. 6) and 426.6 µg/ml (Fig. 7) for PC3, HepG-2 and MCF-7 cell lines respectively.

3. Experimental

3.1. General information

^{1}H NMR spectra were measured by a Bruker 400 MHz NMR spectrometer, at 400 MHz. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d_6 and converted to TMS scale by adding 39.5. Typical conditions: spectral width = 8 kHz for ^{1}H and 30 kHz for ^{13}C, 64 K data points and a flip angle of 45°. FTMS spectra were measured on a Finnigan LTQ-FTMS (Thermo Electron, Bremen, Germany) (Department of Chemistry, Humboldt-Universität, Berlin). UV recording was made on a Shimadzu UV-Visible-1601 spectrophotometer. IR was measured by a JASCO spectra Manager model T/IR-4000 2nd 6000 Series. $(\alpha)_{25}^{D}$ were measured on a Kruess polarimeter -8001 (A. Kruess. Optronic). Paper chromatographic analysis was carried out on Whatman No. 1 paper, using solvent systems: (1) H_2O; (2) 6% HOAc; (3) BAW (*n*-BuOH—HOAc—H_2O, 4:1:5, upper layer). Solvent (2) was used for preparative paper chromatography (PPC).

3.2. Plant material

The leaves of *Cassia grandis* were collected from El-Zohreya Botanical garden, Horticulture Institute, Cairo, in September 2014, voucher specimen (C107) NRC herbarium. Authentication of the collected sample was carried out by Dr. Mona Marzouk, Naional Research Center.

3.3. Extraction and fractionation

Cassia grandis leaves (1.5 kg) were homogenized in a MeOH-H_2O (3:1) mixture (three extractions each with 3 L). The obtained extract was filtered and dried under reduced pressure to give a yield of 32 g dried extract. The obtained extract was re-dissolved in water (500 ml) and extracted with *n*-hexane, methylene chloride, ethyl acetate and *n*-butanol, 500 ml each for three times. Each solvent fraction was collected and dried individually under reduced pressure. The dried fractions were investigated for the phenolic content by two dimensional paper chromatography using BAW as the first solvent and 6% HOAc as the second solvent. Whereas, the *n*-hexane and the methylene chloride fractions were found to contain no phenolic metabolites. The ethyl acetate (1.84 g) and *n*-butanol (9.6 g) fractions were investigated for their phenolic content.

3.4. Folin-Ciocalteau method for the estimation of total phenolics

The phenolic content of the aqueous methanolic leaves extract was estimated by Folin-Ciocalteu reagent and expressed as gallic acid equivalents in µg/mg of crude extract. The Folin-Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic/antioxidant compounds to phosphomolybdic and phosphortungstic acid complexes, which can be determined by measuring the absorbance at 765 nm (Ainsworth and Gillespie 2007).

3.5 Isolation and identification of the phenolic metabolites

The ethyl acetate fraction (1.84 g) was found to contain two major phenolic metabolites of flavonoid nature among a complex matrix of minor components. The ethyl acetate fraction was dissolved in water and applied to a Sephadex LH-20 (Sigma-Aldrich, Sweden) column and eluted with water. The collected sub-fractions were investigated by two dimensional paper chromatography using BAW as the first solvent and 6% HOAc as the second solvent. Compounds **1** and **2** were isolated from sub-fraction II and IV, respectively, by means of preparative paper chromatography. Upon the investigation of the butanol fraction (9.5 g) by two dimensional paper chromatography, it was found to contain three major phenolic metabolites, two of which were found similar to compounds **1** and **2**. The fraction was dissolved in water and applied to a Sephadex column as described above. The same compounds (**1** and **2**) were isolated from sub-fractions X and IX, together with the new natural product (compound **3**) from sub-fraction IV by preparative paper chromatography.

3.6. Spectral data of the new natural product 2- methoxy 6,7,2',6'-tetrahydroxy flavanone 6-O-β- glucoside

Yellow amorphous powder. $[\alpha]_{25}^{D}$ = - 0.40° (c = 0.12 in MeOH). R_f – values: 0.8 (1), 0.85 (2), 0.82 (3). UV λ_{max} (nm) in MeOH: 280. IR (cm^{-1}) in KBr : 3423, 2918, 2850, 1718, 1628, 1453, 1317, 1225, 1074, 1030, 925. ^{1}H-NMR (DMSO, 400 MHz): Table. ^{13}C NMR (DMSO, 100 MHz): Table.

3.7. DPPH radical scavenging assay

Radical scavenging activity of the total extract , the butanol and ethyl acetate fractions against the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically, using the method described by Miliauskas et al. (2004).

3.8. In vitro neutral red cytotoxicity assay

The cytotoxicity assay was performed at the laboratory of cell biology, Faculty of Agriculture, Cairo University. The Neutral Red cytotoxicity assay used was based on the method described by Repetto et al. (2008). Human tumor cell lines tested were HEPG-2 (liver carcinoma cell line), MCF-7 (breast carcinoma cell line) and PC3 (prostate carcinoma cell line).

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Conflicts of interest: None declared.

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