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A sulphated flavone glycoside from *Livistona australis* and its antioxidant and cytotoxic activity

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A new flavone glycoside tricin 7-*O*-β-glucopyranoside-2″-sulphate sodium salt along with 14 known flavonoid compounds were isolated and identified from the aqueous methanol extract of *Livistona australis* leaves. Their structures were established on the basis of extensive NMR (^{1}H, ^{13}C, HSQC and H-H COSY) and ESIMS data. Antioxidant and cytotoxicity properties of the methanol extract of the leaves as well as the new compound were investigated.

Keywords: *Livistona australis*; Arecaceae (=Palmae); sulphated flavone glycoside; *in vivo* antioxidant effect; *in vitro* cytotoxicity

1. Introduction

Arecaceae, also called Palmae, are among the best known and extensively cultivated plant families. *Livistona australis* (R. Br.) Mart. or cabbage-tree palm belongs to the Palmae family. It is a tall, slender palm growing up to about 25 m in height and 0.35 m in diameter. It has leaves plaited like a fan; the cabbage of these is small but sweet and edible (Boland et al., 1984). Some species of the same genus (i.e. *Livistona chinenses*) are used in folk medicine in Southern China for treating various tumours (Ah Lok, 1998). A previous chemical investigation on the leaves of *L. australis* resulted in the isolation of a new pyranone derivative, 3-hydroxy-2-(4-hydroyphenyl)-6-methyl-4-H-pyran-4-one (El-Desouky, Kassem, Al-Fifi, & Gamal El-Deen, 2009). In the course of our phytochemical investigation, a new sulphated tricin glycoside together with 14 known flavonoids were isolated and identified. *In vivo* antioxidant activities of a new glycoside as well as the methanol extract of *L. australis* leaves were evaluated by determination of blood glutathione (GSH) level (Beutler, Duron, & Kellely, 1963). Furthermore, their *in vitro* cytotoxic activities were tested against three human carcinoma cell lines: colon carcinoma *HCT116*, breast carcinoma *MCF7* and liver carcinoma *HEPG2* (Skehan et al., 1990).

2. Results and discussion

2.1. *Identification of isolated compounds*

A new flavone glycoside, tricin 7-*O*-β-glucopyranoside-2″-sulphate sodium salt (**1**), was isolated and identified, together with 14 known flavonoid compounds;

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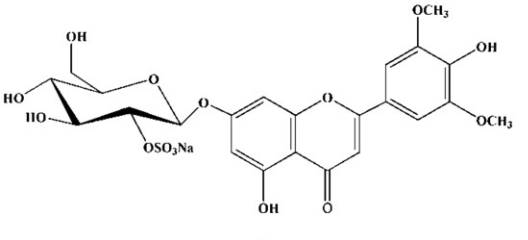
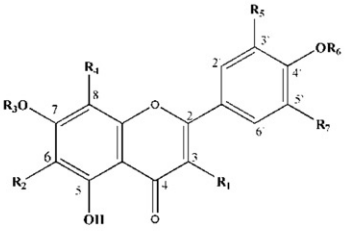
genkwanin-6-*C*-*β*-glucopyranoside (**2**), genkwanin 8-*C*-*β*-glucopyranoside (**3**), isovitexin (**4**), isoorientin (**5**), orientin (**6**) tricin 7-*O*-*β*-glucopyranoside (**7**), tricin 4′-*O*-*β*-glucopyranoside (**8**), luteolin 7-*O*-*β*-glucopyranoside (**9**), quercetin 3-*O*-*β*-glucopyranoside (**10**), qurecetin 3-*O*-*β*-galactopyanoside (**11**), tricin (**12**), quercetin (**11**), apigenin (**14**) and luteolin (**15**). The chemical structures of the isolated compounds were elucidated by extensive UV, NMR and MS spectral data (Figure 1). The identification of known flavonoids **2–15** has been determined by comparing their spectral data with those previously published ones (Ahmed & Shahat, 2006; Guvenalp & Irenzer, 2005; Hasegawa, Tanaka, Hosoda, Takano, & Ohta, 2008; Kim et al., 2010; Kwon, E. Kim, W.J. Kim, W.K. Kim, & C. Kim, 2002).

Compound **1** was obtained as a yellow amorphous powder that exhibits an anionic character on paper electrophoretic analysis. The negative ESIMS spectrum of **1** exhibited ion peak $[M-H]^-$ at 571.14 *m/z* (100%), corresponding to the molecular formula $C_{23}O_{15}H_{23}S$, other significant ion peaks at *m/z* 593.24 $[M+Na\text{-}H]^-$ and 329.4 [M-H-glucose-SO_3]$^-$ confirmed the presence of sodium and sulphate group in the sample molecule. UV spectral data with diagnostic shift reagents indicated a flavone with substituted –OH group at C-7, while those at C-5 and C-4′ are free (Mabry, Markham, & Thomas, 1970; Markham, 1982). ^{1}H-NMR spectrum revealed two equivalent aromatic protons (*δ* 7.33, s, H-2′, H-6′) and two methoxy groups (*δ* 3.85, s, 6H), typical of the 3′, 4′, 5′ trisubstituted ring B of the flavonoid unit. Ring A showed two meta coupled protons resonated at *δ* 6.84 and 6.40 ($J = 1.8$ Hz), each integrated to one-proton, and assigned for C-8 and C-6, respectively. Moreover, one-proton singlet at *δ* 7.0 assigned for H-3 confirmed the flavone nucleus of compound **1**. The presence of anomeric proton of the sugar moiety at a downfield chemical shift (*δ* 5.23, d, $J = 7.5$ Hz) compared to that of tricin 7-*O*-*β*-glucopyranoside data suggested that the *β*-glucose unit should be substituted (Mabry, Markham, & Chari, 1982). The situation of substitution was recognised by the presence of a triplet sugar signal located downfield at *δ* 4.03 experienced by the proton attached to carbon bearing the sodium sulphate substituent (H-2″). The assignment was



1



2: R_1= R_4= R_5= R_6= R_7= H, R_3= CH_3, R_2= Glu
3: R_1= R_2= R_5= R_6= R_7= H, R_3= CH_3, R_4= Glu
4: R_1= R_2= R_5= R_6= R_7= H, R_3= H, R_4= Glu
5: R_1= R_4= R_6= R_7= H, R_3= R_5= OH, R_2= Glu
6: R_1= R_2= R_3= R_4= H, R_5= OH, R_4= Glu
7: R_1= R_2= R_4= R_6= H, R_5= R_7= OCH_3, R_3= Glu
8: R_1= R_2= R_3= R_4= H, R_5= R_7= OCH_3, R_6= Glu
9: R_1= R_2= R_4= R_6= R_7= H, R_5= OH, R_3= Glu
10: R_2= R_3= R_4= R_6= R_7= H, R_5= OH, R_1= O-Glu
11: R_2= R_3= R_4= R_6= R_7= H, R_5= OH, R_1= O-Gal
12: R_1= R_2= R_3= R_4= R_6= H, R_5= R_7= OCH_3
13: R_2= R_3= R_4= R_6= R_7= H, R_1= R_5= OH
14: R_1= R_2= R_3= R_4= R_5= R_6= R_7= H
15: R_1= R_2= R_3= R_4= R_6= R_7= H, R_5=OH

Figure 1. Chemical structures of compounds **1**–**15** isolated from *L. australis* leaves.

also confirmed by ^{1}H–^{1}H COSY experiment, whereby a cross-peak correlated the anomeric glucose (δ 5.23, H-1″) to (δ 4.03, H-2″). The ^{13}C-NMR spectra displayed 23 carbon resonances, 17 of which were assigned to tricin as the aglycone moiety and six for the glucose moiety (Agrawal & Bansal, 1989; Yoon, Kim, & Huh, 2000). Resonances of ring A carbons showed the typical shifts due to the presence of a sugar unit linked to the C-7 hydroxyl group, 1.4 ppm upfield for C-7 and 1 ppm downfield for ortho carbons (C-6 and C-8, respectively) compared to tricin aglycone (Mabry et al., 1982). The presence of a sulphate group linked to the C-2″ of glucose moiety was followed from the upfield shift of the anomeric carbon of glucose moiety at δ 97.9 (4.5) and the corresponding downfield shift of C-2″ signal to 78.98 ppm (3.1) (Nawwar & Buddrus, 1981). The sugar moiety was identified in the pyranose form by comparing its chemical shift values with previously published ones (Agrawal & Bansal, 1989). The assignment of the various sugar carbons was made by 2D-HMQC experiment. The structure was also confirmed by acid hydrolysis; on controlled acid hydrolysis (10% aqueous AcOH, 10 min, 100°C), compound **1** yielded an intermediate which was separated by PPC and identified as tricin 7-*O*-β-glucopyranoside (CoPC, UV, ^{1}H-NMR) (Kwon et al., 2002). Complete acid hydrolysis (2N HCl, 2 h, 100°C) afforded tricin, glucose (CoPC with authentic samples) and sulphate detected by precipitation with barium chloride. The presence of sodium salt was also confirmed by the golden yellow flame test.

Based on the above information, compound **1** was characterised as tricin 7-*O*-β-glucopyranoside-2″-sulphate sodium salt (Figure 1).

2.2. In vivo *antioxidant activity*

Free radical formation and oxidative stress may act as a common pathway to diabetes itself and to its later complications. Hyperglycaemia is accompanied with decrease in GSH level. GSH is a tripeptide normally present at high concentrations intracellularly and constitutes the major reducing capacity of the cytoplasm. It is known to protect the cellular system against toxic effects of lipid peroxidation (Lu, 1999). In this study, the reduced levels of GSH in the diabetic rats were significantly restored by compound **1** and methanol extract of *L. australis* leaves (the percent of change was 3.1% and 18.4%, respectively, compared to 37.46% for the untreated diabetic rats group). Compound **1** is the most potent; it showed activity comparable to that of vitamin E and the plant methanol extract at the tested dose levels (Table 1). This indicates that compound **1** and the methanol extract can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH, or have both effects (Maritim, Sanders, & Watkins, 2003; Rajasekaran, Sivagnanam, & Subramanian, 2005).

Table 1. Effect of methanol extract of *L. australis* leaves, compound **1** and reference drug (vitamin E) on the blood GSH of experimental rats ($n = 6$).

Group	Blood GSH (mg%)	% Change
Control (1 mL saline)	36.3 ± 1.2	–
Diabetic	22.7 ± 0.4*	37.46
Diabetic + vitamine E (7.5 mg kg^{-1})	35.9 ± 0.8**	1.1
Diabetic + methanol extract of *L. australis* leaves (100 mg kg^{-1})	29.6 ± 0.6**	18.4
Diabetic + compound **1** (0.1 mg kg^{-1})	35.2 ± 0.9**	3.1

Notes: Data are expressed as mean ± SEM of 6 rats/group. *Statistically significant difference from control at $p < 0.01$. **Statistically significant difference from diabetic at $p < 0.01$.

2.3. In vitro *cytotoxic activity*

In vitro cytotoxicities (see Section 3.7) of compound **1** and the methanol extract were assayed against liver carcinoma *HEPG2*, breast carcinoma *MCF7* and colon carcinoma *HCT116* cell lines. The American National Cancer Institute assigns a significant cytotoxic effect of extract for future bioguided studies if it exerts an IC_{50} value $\leq 30\,\mu g\,mL^{-1}$ (Stuffiness & Pezzuto, 1990). Compound **1** revealed the highest antiproliferative activity with IC_{50} values of 13.5, 15.2 and $16.5\,\mu g\,mL^{-1}$ against *HEPG2, MCF7* and *HCT116*, respectively, while the methanol extract exhibited less activity against *HEPG2* and *MCF7* cell lines with IC_{50} values of 21.9 and $22\,\mu g\,mL^{-1}$, respectively, and the weakest against colon carcinoma *HCT116* cell line with IC_{50} values of $45.8\,\mu g\,mL^{-1}$.

3. Experimental

3.1. *General experimental procedure*

1-D and 2-D NMR experiments (^1H, ^{13}C, H-H COSY and HMQC) were recorded using a Jeol EX-500 spectrometer: 500 MHz (^1H-NMR), 125 MHz (^{13}C-NMR). The chemical shifts are expressed in (ppm) and the coupling constants in Hz. The mass spectra (ESIMS) were measured on a LCQ Advantage Thermo Finnigan spectrometer; *m/z* (rel.%) and the UV spectra recorded on UV spectrophotometer (Shimadzu model 2401 PC). Column chromatography was carried out on a Polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, Seelze Hanver, Germany) and Sephadex LH-20 (Pharmacia). The paper chromatography (Whatman No. 1 and 3 MM) was performed using solvent systems: (1) H_2O, (2) 15% HOAc (H_2O–HOAc 85:15), (3) BAW (*n*-BuOH–HOAc–H_2O 4 : 1 : 5, upper layer), and (4) BBPW (C_6H_6–*n*-BuOH–pyridine-H_2O 1 : 5 : 3 : 3, upper layer). Solvents 3 and 4 were used for sugar analysis. The compounds were detected by their UV absorption at a wavelength of 254 nm.

3.2. *Plant material*

Fresh plant material was collected from Orman garden, Giza, Cairo, in October 2009. Authentication was performed by Dr M. El-Gibali, former Researcher of Botany at the National Research Centre (NRC). A voucher specimen (no. 124) was deposited in the Herbarium of NRC (CAIRC).

3.3. *Animals*

Adult male albino rats of Sprague-Dawley strain weighing 130–150 g were obtained from the breeding colony of (NRC). The animals were kept under the same hygienic condition, well-balanced diet and water supplied *ad libitum*.

3.4. *Biochemical kit*

Alloxan was purchased from Sigma Co. Cairo, Egypt, vitamin E (dl α-tocopheryl acetate) from Pharco Pharmaceutical Co. Egypt, GSH kit from Wak Co., Germany, and glucose reagent kit was from BioMérieux, France.

3.5. *Extraction and isolation*

Two kilograms of the dried powdered *L. australis* leaves were extracted with 70 : 30 MeOH: water (3×6 L), and the extract was concentrated to dryness under vacuum at 70°C. The residue (200 g) was dissolved in water (500 mL). The aqueous solution was successively extracted with n-hexane (2×500 mL) for defatting. The defatted methanol extract was subjected to polyamide 6S column chromatography ($125 \times 5\,cm^2$), eluting with

MeOH/H_2O mixtures of decreasing polarities to yield five main fractions. Fraction I (20% MeOH/H_2O), chromatographed on PC using BAW (double solvent) two times and then purified on a Sephadex LH-20 column using methanol, yielded compound **1** (35 mg). Fractions II–V afforded compounds **2**–**15**. Their isolation was achieved by a combination of smaller polyamide column ($75 \times 2.5\,cm^2$) and PPC using H_2O, 15% HOAc and BAW as eluents and then purified on Sephadex LH-20 column using methanol as eluent.

3.5.1. *Tricin 7-O-β-glucopyranoside-2″-sulphate sodium salt (**1**)*

Yellow amorphous powder, *Rf*-values: 0.92 (H_2O), 0.87 (HOAc), 0.45 (BAW). Electrophoretic mobility: 1.8 cm, on Whatman no. 3 MM paper, buffer solution of pH 2, H_2O–AcOH–HCOOH (89.5 : 8 : 2.5), 90 min, 50 V cm^{-1}. Negative ESIMS *m/z*: $[M-H]^-$ 571.14 (100%) corresponding to a molecular formula $C_{23}O_{15}H_{23}S$ and $[M+Na\text{-}H]^-$ 593.24 ($C_{23}O_{15}H_{23}S$ Na). UV λmax nm in MeOH: 283, 349; MeOH/NaOMe: 280, 425; $AlCl_3$: 287, 318, 362, 404; $AlCl_3$/HCl: 289, 316, 362, 403; NaOAc: 283, 349, 419; NaOAc/H_3BO_3: 281, 360. ^{1}H-NMR, 500 MHz in DMSO-d_6: δ 7.33 (2H, s, H-2′, H-6′), 7.00 (1H, s, H-3), 6.84 (1H, d, $J = 1.5$ Hz; H-8), 6.40 (1H, d, $J = 1.5$ Hz; H-6), 3.85 (6H, s, 2 [OCH_3]), 5.23 (1H, d, $J = 7.5$ Hz; H-1″), 4.03 (1H, t, H-2″), 3.70 (1H, m, H-6″), 3.60 (1H, t, H-3″), 3.42 (1H, m, H-5″), and 3.22 (1H, m, H-4″). ^{13}C-NMR, 125 MHz in DMSO-d_6: δ 182.6 (C-4), 164.7 (C-2), 163.2 (C-7), 161.7 (C-5), 157.3 (C-9), 148.8 (C-3′,5′), 140.6 (C-4′), 120.7 (C-1′), 105.9 (C-3), 105.0 (C-2′,6′), 104.3 (C-10), 99.8 (C-6), 97.9 (C-1″), 96.1 (C-8), 79.0 (C-2″),77.5 (C-5″), 76.5 (C-3″), 70.1 (C-4″), δ 61.1 (C-6″) and δ 56.9 (2 OCH_3).

3.6. *Determination of antioxidant activity*

The antioxidant activities of the methanolic extract of *L. australis* and compound **1** were determined by measuring the GSH level in blood of alloxan-induced diabetic rats upon administration of extract and compound **1** for one week (Beutler et al., 1963; Eliasson & Samet, 1969). Thirty rats were divided into five groups (six animals each). One group was kept as a negative control while diabetes was induced in the other groups by a single intraperitoneal administration of alloxan at a dose of 150 mg kg^{-1} bodyweight (BW) followed by an overnight fast. Blood samples were collected from the retro-orbital venous plexus of each rat and the blood glucose level was measured to confirm induction of diabetes using BioMérieux kits (Trinder, 1969). Diabetic rats were divided into four groups. The first group was kept untreated, and the second and third groups were given the methanol extract of *L. australis* and compound **1** in oral doses of 100 and 0.1 mg kg^{-1} BW, respectively, for 7 days. The last group received the reference drug (vitamin E, 7.5 mg kg^{-1} BW in an oral dose of 100 mg kg^{-1} BW for 7 days.). At the end of the experiment, blood samples were obtained and blood GSH level was measured using GSH kit [Elman's reagent, 5, 5-dithio bis-(2-nitrobenzoic acid), to yield a stable yellow colour which can be measured colorimetrically at 412 nm]. The intensity of the yellow colour developed is related to the amount of GSH in blood. Results were presented as the mean ± SEM. Student's *t* test was used to analyse statistical significance (Table 1).

3.7. *Determination of cytotoxic activity*

Compound **1** as well as the methanol extract of *L. australis* were screened for cytotoxicity using the method of Skehan (Skehan et al., 1990). Cells were plated in 96-multiwell plate (104 cells/well) for 24 h before treatment with the extract to allow attachment of the cell to the wall of the plate. Different concentrations of the tested substance (0, 1, 2.5, 5 and 10 μg mL^{-1}) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with compounds for 48 h at 37°C in an

atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained with sulphorhodamine B stain. Excess stain was washed with acetic acid and the attached stain recovered with Tris EDTA buffer. Colour intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration was plotted to obtain the survival curve of each tumour cell line as compared with doxorubicin (the control anticancer drug).

4. Conclusion

In this study, a new sulphated flavone glycoside **1**, along with 14 known flavonoid compounds, were characterised for the first time from *L. australis* leaves. Compound **1** and the methanol extract of the plant leaves revealed a remarkable biological activity in restoring the reduced GSH levels in diabetic rats, which could be attributed to their antioxidant activity or their role in increasing the biosynthesis of GSH. Compound **1** also exhibited a more potent cytotoxic activity against *HEPG2, MCF7* and *HCT116* than the methanol extract of the plant leaves.

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