Isolation of Cardenolide glycosides from *Pergularia tomentosa.L* and their Antioxidant activities

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ABSTRACT: Continuing our investigations on plants belonging to the Asclepiadaceae family, five known cardenolide glycosides (1-5), have been isolated from the aerial parts of *P. tomentosa.L.* The structures of these isolated compounds were identified by spectroscopic data as Uscharidin 1 which is isolated for the first time from *Pergularia tomentosa.L,* Calactin 2, 12 β-hydroxycalotropin 3, 6'-hydroxycalactin 4, and Galakinoside 5. The structures were elucidated by chemical and spectroscopic methods (2D-NMR, IR, and MS) and by comparison with the authentic samples (literature data). In addition, antioxidant activities of three cardenolide, the highest scavenging activity of DPPH, was observed in 2 (IC50= 1953 µg/ ml), followed by 4 (IC50= 3384 µg/ ml), and 3 (IC50= 3410 µg/ ml).

KEYWORDS: *P. tomentosa.L,* Asclepiadaceae, cardenolide, 2D-NMR, antioxidant activities.

1. Introduction

The family Asclepiadaceae is a very rich source of the cardenolide cardiac glycosides [1]. *Pergularia tomentosa* L. known locally as Ghalaka [2], a climbing to semierect perennial herb, belongs to the milkweed family [3]. Our previous work was concerned mainly with the isolation and characterization of new triterpene from the ethanolic extract [4]. Since cardenolides are active against a large range of cancer cell types [5, 6].

Previous studies reported the presence of the cardenolide glycosides ghalakinoside, calactin, uzarigenin, and pergularoside in the roots of *Pergularia tomentosa L* and desglucouzarin, coroglaucigenin, and uzarigenin along with a *β*-sitosterol glucoside in the leaves [1, 5], ghalakinoside was identified as a cytotoxic cardiac glycoside [2] and a potential antitumor agent [6, 7]. In our ongoing research for new compounds from medicinal plants from Algerian flora [8], we have investigated the ethanolic extract of the aerial parts of *P. tomentosa.L*. Here we report the occurrence of five known cardenolide glycosides uscharidin **1** [9], Calactin **2**, 12β-hydroxycalotropin **3**, 6'-hydroxycalactin **4**, and Galakinoside **5.**

In this paper, we describe the antioxidant activities of three cardenolide **2- 4**. The antioxidant capacity was evaluated by DPPH· radical.

2. Results and discussion

The crude ethanol extract of the air-dried (7.0 kg), ground, and defatted plant material (254 g) was partitioned, according to the procedure described in the experimental section, into chloroform-soluble and water soluble extracts. The water soluble extracts was further extracted with *n*-butanol. The butanol extract (24 g) was subjected to repeated silica gel column chromatography (CC) with dicloromethane the polarity was then increased gradually by adding methanol that yielded compounds 1–5.

Compound **1** (figure 1) was obtained as oil, molecular formula was unequivocally established as $C_{29}H_{38}O_9$ by HRMS displayed a molecular ion peak at m/z 529 [M-H^+]. The IR spectrum of this compound **1** showed a broad band at 3400.29cm^{-1}due to hydroxyl groups and a strong band at

1733.83 cm^{-1} attributed to the carbonyl. Also the spectrum showed a band at 1624.18 cm^{-1} characteristic of a double bond group.

2D- NMR experiments, including $^{1}H–^{1}H$ COSY, HMQC and HMBC, were used to assign the relations between ^{1}H and ^{13}C NMR resonances. Examination of the ^{1}H NMR spectrum of **1** confirmed its steroidal nature and the presence of the characteristic α,β-unsaturated lactone of a cardenolide. The latter group was represented by a broad singlet at δ 5.83 (l H,s, 22-H) and an AB quartet further split by the 21-H at δ 5.77 (2H, 21-H, J=9.51) and 5.05 (1H, dd, *J*=7.67*)*, and two methyl signal at 0.91 (3H, s) and 0.81 s, signal at *δ* 9.88 (3H, s), ascribable to an aldehyde group. Additionally, the ^{1}H- NMR spectrum of **1** displayed a signal corresponding to an anomeric proton at *δ* 4. 49 (1H, s), which suggested that **1** is a doubly linked. The ^{13}C NMR spectrum of **1** (Table 1) showed 29 carbon signals, of which 23 were assigned to the aglycone moiety and 6 to a sugar portion. The ^{13}C NMR chemical shifts of all the hydrogenated carbons could be unambiguously assigned by the HMQC spectrum.

The ^{13}C – NMR spectrum of **1** showed the presence of two carbonyls at δ 209.40(C19) and at 212.26 (C3') along with resonances typical of a butenolactone ring: a carbonyl group (δ 170.2), an olefinic quaternary function (δ 174.3), an olefinic methine (δ 117.9), and an oxymethylene group (δ 73.4) and two methyl function at *δ* 21.59(C6') and 9.63 (C18).

In the HMBC spectrum, long-range correlations were observed from this aldehyde proton signal to the C-1 (δ 38.4), C-5 (δ 49.18) and C-9 (δ 45.37). ^{13}C NMR data (Table 1) of **3 (**12 β-hydroxycalotropin) (figure1) closely resembled those of Calactin **2** and showed that compound **3** differed from **4** only by the absence of the C-12 hydroxyl group.

The chemical shifts of C-11 (δ C 27.53 for 2, δ C 22 for 1) were moved downfield dramatically. These features implied the hydroxyl group was linked at C-12. The α-orientation of H-17 was assigned on the basis of the coupling constants of the signal at δ 2.85 (2.76, dd, (J= 5.60) [1,5].

Several methods have been developed to evaluate the antioxidant activity, using the quenching of synthetic or generated radicals in polar organic solvents such as methanol [8]. In this study, the DPPH· and regenerated was used to assess the antiradical activities of the cardenolide glycosides. The IC50 values for the DPPH free radical scavenging activities of the compounds are shown in Figure 2 in compared to ascorbic acid. As shown in Fig. 2, compared to ascorbic acid (IC50=9.5 µg/ ml), the highest scavenging activity of DPPH was observed in **2** (IC50= 1953 µg/ ml), followed by **4** (IC50= 3384 µg/ ml), and **3** (IC50= 3410 µg/ ml). The capability of compound to scavenge DPPH was assessed on the bases of their IC50 values which defined as the concentration of test compound that decreases the concentration of DPPH free radical to half of its initial value. As shown in Fig. 2, these compound did not showed good free radical scavengers compared to ascorbic acid (IC_{50}=9.5 µg/ ml), but general speaking we can say that the ability of **1** to scavenge DPPH free radical was more effective than that of **4** and **3** (IC_{50} = 3384 and 3410, respectively). Therefore, compound **2** could be act as good source for antioxidant industries.

3. Experimental

3.1. General experimental procedures

The IR spectra were recorded on Thermo-Nicolet Nexus 870 FT-IR spectrophotometer (Thermo Scientific, Wisconsin, USA). ^{1}H-NMR spectra were recorded on a Bruker DPX-300 MHz spectrometer with TMS as an internal standard. ^{13}C-NMR spectra were recorded at 75.5 MHz (Strasbourg, France). HRMS were measured in positive ion mode using electrospray ionization (ESI) technique on a Bruker APEX-2 instrument (Bremen, Germany). Column chromatography (CC) was performed on silica gel 60 (0.063-0.200 mm, Fluka, Steinheim, Germany) or silica gel S (Ridel de-haën, Seelze-Hannover, Germany). Purification of the compounds was achieved by routine Thin Layer Chromatography (TLC) on silica gel G-UV_{254} glass plates (0.25 mm, Macherey-Nagel, Easton, PA, USA). Compounds were visualized by spraying with sulfuric acid – anisaldehyde spray reagent followed by heating at 120 °C Spectrophptometer (Novaspec 11, LKB Biochrome, England).

3.2. Plant material

Pergularia tomentosa.L was collected from the surroundings of Ghardaia (South Algeria) during the flowering period (April 2008). The plant was identified by Prof. Dawud AL-Eisawi (Department of Biological Sciences, Faculty of Science, University of Jordan). A voucher specimen of the plant (BAU/08/AP-Alg) is deposited at the Herbarium of the Department of Biological Sciences, University of Jordan.

3.3. Extraction and isolation

The ground whole plant material (7.0 kg) was defatted by soaking in petroleum ether at room temperature and then repeatedly soaked in ethanol at room temperature (50 L, 4 times, 5 days each). The residue obtained upon removal of EtOH under reduced pressure (255 g) was partitioned between water and chloroform. The water soluble fraction was extracted with *n*-butanol. The butanol extract (24) was subjected to repeated silica gel column chromatography (CC) with dicloromethane the polarity was then increased gradually by adding methanol that yielded compounds 1–5. Each fraction was purified by a combination of CC, TLC and recrystallization.

The butanol extract (CB) (25 g) was adsorbed on 50 g silica gel S and subjected to column chromatography (50×5.6 cm) using 500 g of the same adsorbent. The column was packed in dichloromethane and the polarity gradually increased using methanol, till pure methanol was added. A total of 30 fractions (500 ml each) were collected and grouped into six groups (PBI – PBVI) according to their TLC behavior.

Fraction PBII (4.5 g) offered **2.** Fraction PBIII (6.3 g) give **3** (1g) and 4 (1g). Fraction PBIV (1 g) offered **5** (700mg). Fractions (PBV) give **1** (810mg).

3.4. Antioxidant activity [9]:

Determination of DPPH radical-scavenging activity:

DPPH (1, 1-diphenyl-2-picrylhydrazyl) was used to determine the free radical scavenging activity of the compoumds by a method of Bloiss method (1958). DPPH radical was freshly prepared by dissolving 0.0024g DPPH in 100ml methanol (purple). To 1 ml of different concentrations from *10µg/ml - 200µg/ml, 1ml DPPH was added and left in dark for 20 mints, and then the absorbance was read at 517 nm* with single beam spectrophotometer. Ascorbic acid was used as a positive control. The percentage inhibition of DPPH formation was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \text{ X } 100$$

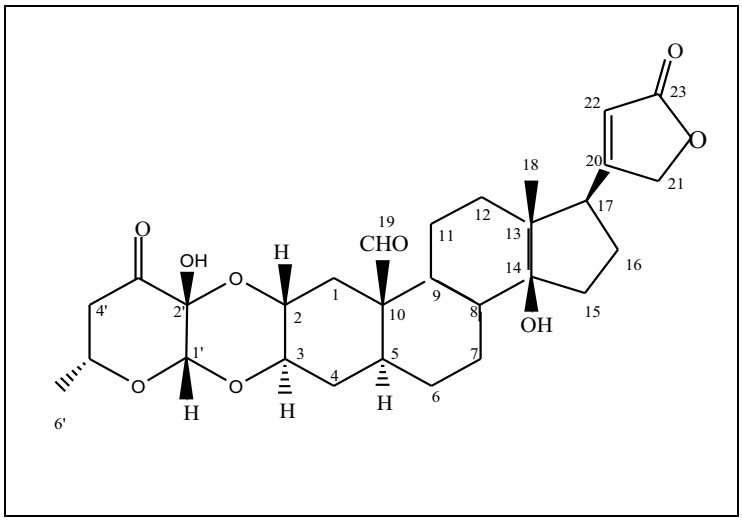
Calculation of 50% Inhibitory Concentration (IC50):IC50 value (the concentration of extract in µg/ml required to scavenge 50% of the DPPH free radical) was calculated from the inhibition curve using the percentage scavenging activities or percentage inhibition at five different concentrations of the compound.

12β-Hydroxycalotropin 3: yellow oil, m.p 150-155°C Rf value 0.62 blue spot (2%Methanole\Chloroforme). The IR (KBr)(cm^{-1}): 3435.27 (OH), carbonyl 1729.52, 1462.03 (C=C). Both the ^{1}H and ^{13}C NMR (DMSO-d6): δ 5.86 (1H, s, 22-H), 5.77 (2H, 21-H), 4.88 (1H-1',s, J=10.04), 9.89 (CHO); δ 1.51 (3H, H-18). The ^{13}C – NMR (DMSO-d6) oxymethine at 76.79 (C-12) and C-18 (δ 12.40), an olefinic methine at δ 116.69, and an oxymethylene group at δ 72.88 ppm

Uscharidin 1: yellow oil, m.p 210-215°C (literature value 290°C Hesse, 19), Rf value 0.57 (2% Methanole\Chloroforme).The IR (KBr) (cm^{-1}): 3400 (OH), carbonyl 1733.83, 1624.18 (C=C). MS m\z (%): 529 (21.1 %)[M + H^{+}], 517.37 (19.4)[M^{+}- C H_3]. ^{1}H NMR (DMSO-d6) :δ 5.83 (l H, 22-H) and an AB quartet further split by the 22-H at δ 5.77 (2H, 21-H,), δ 9.88 (CHO). The ^{13}C – NMR (DMSO-d6) 212.26 (CHO), δ 209.55(CO) and 176.84(C-23); δ 177.21(CO), an olefinic methine at δ 116.69, and an oxymethylene group at δ 73.64 ppm.

Table 1: ^{13}C NMR (75 MHz) and ^{1}H NMR (300 MHz) Data of Compounds 1 and ^{13}C NMR (75 MHz) of 2; 4

		5	**4**	**2**
	δ_H(J in Hz	δ_C (J in Hz)	δ_C (J in Hz)	δ_C (J in Hz)
1	2.35 dd	38.4, CH_2	38.52	35.55
	(12.4,4.5)			
	1.86 (m)			
2	5.0 (m)	70.66 , CH	70.48	70.48
3	3.7 dd (10.3)	72.84 , CH	72.72	72.72
4	1.58 d (14.3)	27.9 , CH_2	32.55	27.90
5	1.60 m	49.18 , CH	45.59	42.14
6	2.3 m	27.7 , CH_2	27.70	27.7
	1.84 m			
7	1.23 m	28.7 , CH_2	26.81	27.80
8	1.62 m	42.14 , CH	40.43	41.64
9	1.45 m	45.37, CH	44.42	49.46
10		50.56 , qC	40.71	50.61
11	1.85 m	23 , CH_2	31.76	27.53
12	1.68 m	34.50, CH_2	73.64	76.79
13		50.29, qC	50.67	49.83
14		84.33, qC	84.35	84.0
15	2 .23, t (4.46)	32.50, CH_2	32.50	32.5
16	2.21 m	27.60, CH_2	27.48	27.60
	1.9 m			
17	2.76, dd (5.60)	54.66, CH	46.43	50.61
18	0 .91 s CH_3	9.63, CH_3	16.29	12.40
19	9.88 s	212.26, CH	61.51	211.92
20		177.21, qC	177.16	177.16
21	5.77 (1.4)	73.64, CH_2	72.92	72.88
	5.05 dd (7.67)			
22	5.83 s	116.76 , CH	116.69	116.60
23		176 , qC	174.41	174.41
1’	4 .49 s	95.67 , CH	96.25	92.02
2’		103.36 , qC	101.04	94.27
3’		209.55 , qC	72.71	84.23
4’	1.66 m	40.44 , CH_2	32.55	27.50
5’	4.19 t (2.67)	68.83 , CH	72.36	69.39
6’	0.81 CH_3	21.59 , CH_3	63.49	21.43



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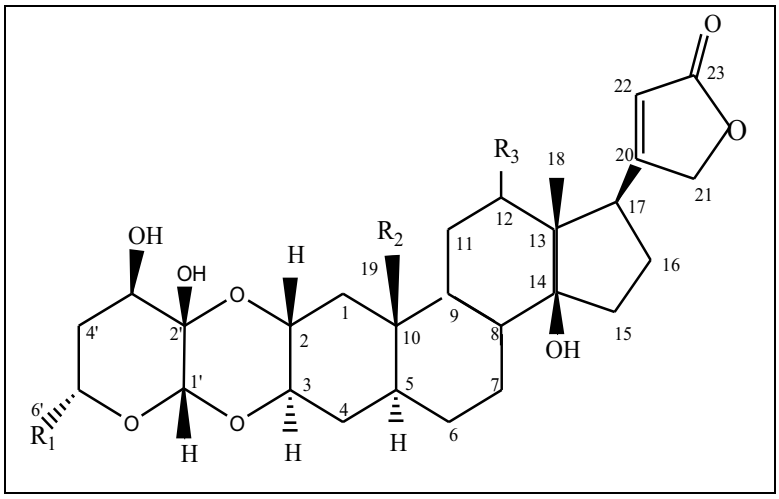

2	$R_1 = CH_3$	R_2= CHO	R_3= H
3	$R_1 = CH_3$	R_2= CHO	R_3= OH
4	$R_1 = CH_2OH$	R_2= CHO	R_3= H
5	$R_1 = CH_2OH$	R_2= CH_2OH	R_3= H

Figure 1: Chemical structures of cardenolide isolated from *Pergularia tomentosa L*

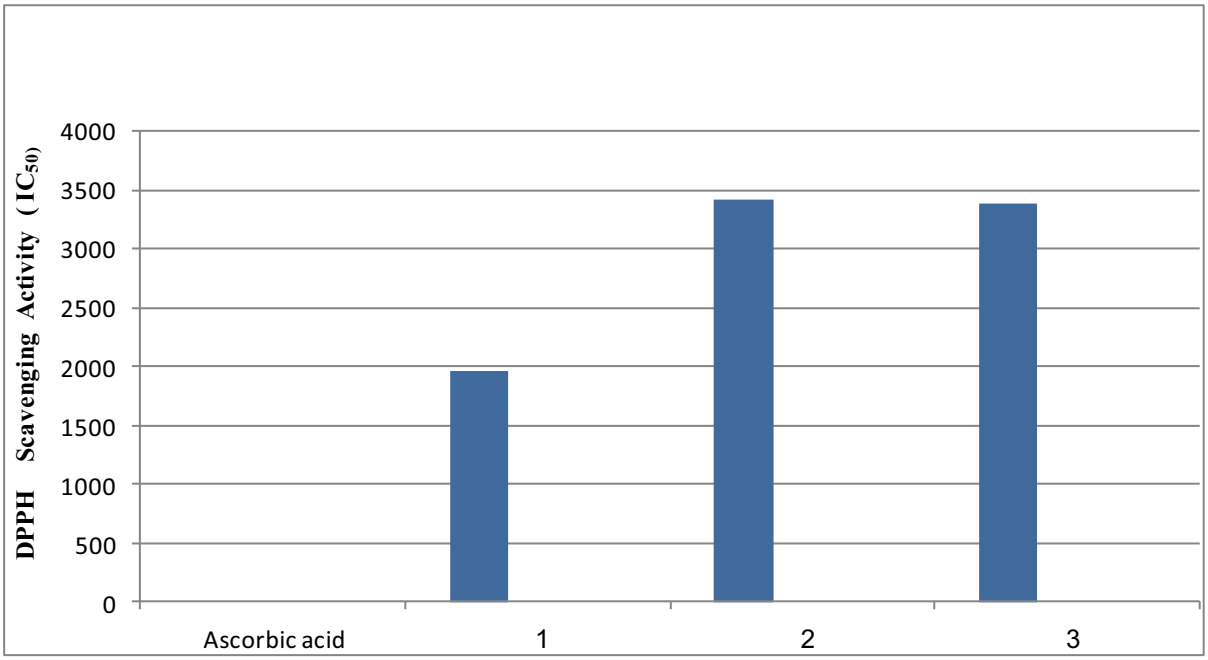

Figure 2: DPPH Scavenging Activity (IC50) of Ascorbic acid and tested compounds

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