Chemical constituents of the aerial parts of Algerian *Galium brunneum*: isolation of new hydroperoxy sterol glucosyl derivatives

Abla Smadi[a,,b], Fatma Bitam[a,c], Maria Letizia Ciavatta[a,*], Marianna Carbone[a], Anis Bertella[d], Margherita Gavagnin[a]

Affiliation

[a] *Consiglio Nazionale delle Ricerche (CNR), Istituto di Chimica Biomolecolare (ICB), Via Campi Flegrei, 34 -80078- Pozzuoli, Napoli, Italy*

[b] *Faculté des Sciences de la Matière, Département de Chimie, Laboratoire de chimie et Chimie de l'environnement (LCCE),Université de Batna 1, 05000, Algérie*

[c] *Faculté de Médecine, Département de Pharmacie, Université de Batna 2, 05000, Algérie*

[d]*Faculté des Sciences de la Matière et de la Vie, Départment de Biologie, Laboratoire de la Microbiologie Appliquée, Université d'Oran 1, 31100, Algérie*

*****Corresponding author**
Email*:* lciavatta@icb.cnr.it (M. L. Ciavatta)

Abstract

The liposoluble extract of *Galium brunneum* aerial parts from North-eastern Algeria was chemically investigated. The EtOAc soluble portion contained a series of glycosyl cucurbitacins and sterols including three new glucosyl hydroperoxy sterols **1–3** among other phenolic components whereas the BuOH soluble fraction was dominated by glycosyl derivatives of flavonoids, iridoids and lignans, according to the chemistry reported in the literature for the genus *Galium*. The structure of new oxidized sterols **1–3** was determined by spectroscopic methods as well as by comparison with related known metabolites. Selected main compounds from both extracts, which revealed moderate antibacterial activities, were tested for their growth inhibitory properties against Gram-positive and Gram-negative bacteria. This is the first report of cucurbitacins in plants of genus *Galium*.

Key words

1. Introduction

The Rubiaceae family is one of the largest in the Magnoliopsida class with a cosmopolitan distribution (Robbrecht, 1988). According to recent phylogenetic molecular studies, this family is divided into three subfamilies, Rubioideae, Cinchonoideae, and Ixoroideae, including numerous tribes due to high species diversity (Bremer, 2009). The genus *Galium* consisting of about 400 herbaceous plant species represents a large genus of the subfamily Rubioideae (tribe Rubieae) that are world-wide distributed, mostly from the tropics to the temperate zones (Ehrendorfer et al., 2018; Ehrendorfer et al., 1976; The Plant List, 2013). Many *Galium* species are used in traditional medicine to treat various pathologies such as epilepsy, hepatitis, phlebo phlogosis, kidney disorders and skin infections and as diuretic and analgesic (Jarić et al., 2007; Chinese Materia Medica, 1977; Bolivar et al., 2011; Shah et al., 2006; Kaval et al., 2014). A large number of phytochemical investigations have been conducted on several *Galium* species from different geographical areas including Algeria. These studies have reported the occurrence of iridoids, triterpenoids, anthraquinones, lignans and flavonoids displaying a variety of interesting biological (Martins et al., 2015; Bradic et al., 2018; Mocan et al., 2016; Chaher et al., 2016; Camero et al., 2018; Gaamoune et al., 2014).

Galium brunneum Munby is a species with a few records limited to North Africa (Algeria, Morocco and Tunisia). The plant is a perennial herb growing on calcareous rocks and is characterized by brown or reddish flowers. Inflorescences are grouped in short and axillary cymes, in the upper portion of the stems (Quezel and Santa, 1963). Neither phytochemical nor pharmacological studies have been reported in the literature to date for this plant.

In continuing our recent phytochemical investigations on Algerian plants (Bitam et al., 2008; Bouzergoune et al., 2016; Boumaraf et al., 2017; Zergainoh et al., 2018; Djebara et al., 2019), we have analyzed the content of EtOAc and BuOH soluble parts from the hydromethanolic extract of the aerial parts of *G. brunneum* collected in Ain Touta region, Batna (Algeria) in May of 2012

(Smadi, 2018). The chemical study revealed a secondary metabolite pattern characterized by flavonoids, iridoids and lignans, according to the chemistry reported in the literature for the genus *Galium*. Three unreported hydroperoxy glycosyl sterols (**1-3**, Fig. 1) were found in the EtOAc extract along with a series of known compounds (**4-14**, see Supplementary material). Among them known glycosyl triterpenoids (compounds **7**, **8**, and **10**) with cucurbitane nucleus, that was never reported to date in genus *Galium*, were identified. Previously reported compounds (**15-25**, see Supplementary material) were isolated from the BuOH extract. Due to moderate antibacterial properties exhibited by the *G. brunneum* extracts against Gram-positive and Gram-negative bacteria strains, selected main metabolites **11** and **13** from EtOAc and compounds **15**, **16**, and **18–22** from BuOH extracts were evaluated for this activity.

2. Results and discussion

The extracts of dried aerial parts of *G. brunneum*, obtained as described in details in Experimental, were analyzed by TLC chromatography using different eluent systems. Only EtOAc and BuOH extracts were considered in this study. Aliquots of these extracts were submitted to a series of sequential fractionation steps on SiO_2, LH-20, C18 chromatography as well as HPLC (see Experimental). Twenty-five pure compounds belonging to different structural classes and including three unprecedented hydroperoxy sterols **1-3** (Fig. 1) were finally obtained.

Known metabolites **4–25** were identified by comparison of their spectroscopic data (NMR, MS and optical rotation) with the literature as: syringaresinol (**4**) (Dickey, 1958; Abe and Yamauchi, 1988), balanophonin (**5**) (Haruna et al., 1982; Sy and Brown 1999), lariciresinol (**6**) (Badawi et al.1985; Wang et al., 2010), hexa-*nor*-cucurbitacin D 2-*O*-β-D-glucoside (**7**) (Chen et al., 2009), deacetoxycucurbitacin B 2-*O*-β-glucoside (**8**) (Stuppner and Wagner, 1989), β-sitosterol-3-β-*O*-D-glucoside (**9**) (Sakakibara et al., 1983; Cheng et al., 1992), cucurbitacin D 2-*O*-β-D-glucoside (**10**) (Yamada et al., 1977), phlorizine (**11**) (Rui-Lin et al., 1982), pinellic acid (**12**)

(Nagai et al., 2002), daphyllosid (**13**) (Demirezer et al., 2006), quercetin (**14**) (Mabry et al., 1970), 10-*O*-*trans*-*p*-coumaroyl-scandoside (**15**) (Otsuka et al., 1991), asperulosid (**16**) (Otsuka et al., 1991), deacetyl asperulosidic acid (**17**) (El-Naggar and Beal, 1980), an inseparable mixture of dihydro-dehydro-diconiferyl alcohol 4-*O*-β-D-glucoside (**18**) (Ouyang et al., 2011) and dehydro-diconiferyl alcohol 4-*O*-β–D-glucoside (**19**) (Salama et al., 1981), isorhamnetin 3-*O*-β-rutinoside (**20**) (Ogawa et al., 2001), quercetin 3-*O*-β-rutinoside (**21**) (Mabry et al., 1970), phloretin 3',5'-di-*C*-β-D-glucoside (**22**) (Ogawa et al., 2001), (7*S*,8*R*,8'*R*)-(-)-lariciresinol 4-4'-bis-*O*-D-glucoside (**23**) (El-Gamal et al., 1997), deacetylasperulosid (**24**) (Otsuka et al., 1991), and, finally, deacetyldaphyllosid (**25**) (Otsuka et al., 1991). The structure of unprecedented hydroperoxy sterols **1–3**, established by detailed spectroscopic analysis, mainly 1D and 2D-NMR techniques, was described as follows.

2.1 Structure elucidation of compounds 1-3

Preliminary ^{1}H-NMR analysis of compounds **1–3**, conducted in both pyridine-d_5 and methanol-d_4, clearly indicated that all three compounds belong to the sterol class being oxidized derivatives of co-occurring β-sitosterol-3-β-*O*-D-glucoside (**9**). The HR-ESI-MS spectra of **1–3** showed the same sodium adduct ion peak at 631.4187 *m/z* (M+ Na)$^+$ corresponding to the molecular formula $C_{35}H_{60}O_8$ with two oxygen atoms more with respect to sterol **9** thus suggesting the presence of a hydroperoxy function in their structures. Comparison of ^{1}H- and ^{13}C-NMR spectra (Tables 1 and 2) confirmed the presence of an additional hydroperoxy function in all three compounds and revealed that the structures of **1–3** differed from **9** only in the fragment C-4/C-7 being the remaining part of the molecules the same as **9**.

Compound **1** was determined to be (24*R*)-24-ethyl-7α-hydroperoxy-cholest-5-en-3-*O*-β-D-glucopyranoside. The double bond was located at C-5/C-6 the same as **9** and the hydroperoxy substituent was positioned at C-7 as it was easily evidenced by analysis of the ^{1}H-^{1}H COSY

spectrum. In fact, the sequence H_2-1/H_2-4 was the same as in **9** whereas in ring B an additional deshielded proton at δ_H 4.36 (H-7) attached to a carbon bearing the –OOH substituent was observed to be coupled with both olefinic proton H-6 (δ_H 6.07, dd, J = 4.7, 1.5 Hz) and angular methine H-8 (δ_H 1.60, m) (Table 1). Consistent with this, the ^{13}C-NMR spectrum (Table 2) of **1** contained a CH signal at δ_C 77.8 (C-7) replacing the CH_2 signal at δ_C 32.0 observed in the carbon spectrum of **9**.

The α-orientation of 7-OOH was inferred by analysis of H-7 multiplicity (dd, J = 4.0, 4.0 Hz) which is consistent with an equatorial orientation. In addition, the ^{13}C-NMR values of C-9 (δ_C 43.9) and C-14 (δ_C 49.4) appeared significantly at low ppm with respect to the corresponding carbons in β-sitosterol-3-β-O-D-glucoside (**9**) (δ_C 50.0 and 56.9, respectively) (Table 2) due to expected γ-gauche effect of the axial substituent at C-7. The ^{1}H- and ^{13}C-NMR assignment of **1** was in agreement with that reported for the corresponding 7α–OH derivative (Chaurasia and Wichti, 1987; Agnihotri et al., 2008).

Compound **2** was identified as (24R)-24-ethyl-5α-hydroperoxy-cholest-6-en-3-O-β-D-glucopyranoside. It exhibited the $\Delta^{6,7}$ disubstituted double bond and 5–OOH group as it was indicated by two double doublets of an AB system at δ_H 5.92 (J = 10.0 and 2.5 Hz, H-6) and δ_H 5.68 (J = 10.0 and 1.5 Hz, H-7), in the ^{1}H-NMR spectrum, as well as two CH signals at δ_C 131.8 (C-6) and 133.6 (C-7) and a C signal at δ_C 82.8 (C-5), in the ^{13}C-NMR spectrum. This arrangement was confirmed by diagnostic HMBC correlations between C-5 and H_3-19 (δ_H 0.92), H_2-1b (δ_H 1.30), H_2-4a (δ_H 3.16), H-6 (δ_H 5.92) and H-7 (δ_H 5.68). The α-configuration of 5-OOH substituent was suggested from the signals observed in the ^{13}C-NMR for C-6 (δ_C = 131.8) and C-7 (δ_C = 133.6) that were comparable with those reported for the corresponding 5α-OH derivative (Agnihotri et al., 2008; Holland and Jahangir, 1983).

The structure of compound **3** was established as (24R)-24-ethyl-6β-hydroperoxy-cholest-4-en-3-O-β-D-glucopyranoside. The sequence H_2-1/H-4 was easily deduced by ^{1}H-^{1}H COSY experiment

allowing the location of the double bond at C-4. In fact H-3 (δ_H 4.48, m) showed a diagnostic vicinal correlation with vinyl proton H-4 (δ_H 6.18, br s) along with the expected coupling with H_2-2 (δ_H 2.16 and 1.90). The hydroperoxy substituent was positioned at C-6 (δ_C 75.0, δ_H 4.63) as it was evidenced by analysis of COSY spectrum where H-6 was coupled to methylene H_2-7 (δ_H 1.92 and 1.11) further correlated to angular methine H-8 (δ_H 1.65). Long-range correlations observed in the HMBC spectrum between quaternary sp^2 carbon C-5 (δ_C 143.3) and H_2-1 (δ_H 1.82 and 1.02), H-3 (δ_H 4.48), and H_3-19 (δ_H 1.33) as well as between C-4 (δ_C 129.2) and H-6 (δ_H 4.63) supported the proposed structure. The β-orientation of 6-OOH was suggested by analysis of the coupling constants of H-6 which resonated as an apparent triplet (J = 3.0 Hz) according to its equatorial orientation. Consistent with this, the spatial effect of β-oriented 6-OOH was observed on the chemical shift values of H_3-19 (δ 1.33) as it was evident by comparing NMR values of model compounds exhibiting with 6α–OH or 6β–OH substituent (Shi et al., 2008; Zhao et al., 2005; Zhao et al., 2003). In addition, due to the γ-gauche effect of the axial substituent at C-6, the ^{13}C-NMR value of C-8 appeared at δ 30.1, in agreement with the carbon values reported for 6β–OH related compounds.

The configuration of the anomeric carbon of compounds **1-3** was defined as β from the coupling constant value of H-1' (J = 7.6–7.8 Hz) for all of them. Biogenetical considerations as well as comparison of the ^{13}C-NMR values of side chain carbons of **1–3** with those of **9** (Table 2) led us to assign the same 24R absolute configuration. The configuration at C-24 of **9** was established by analysis of the carbon chemical shifts in chloroform-d of the aglicone obtained by MeOH/HCl hydrolysis of **9** that well fitted with the (24R)-epimer of β-sitosterol (Wright et al., 1978). Full assignment of proton and carbon NMR values of **1–3** was made by 2D-NMR experiments and reported in Tables 1 and 2 (in pyridine-d_5) and in Experimental (in methanol-d_4).

It should be noted that aglycone hydroperoxy sterols from compounds **1** and **2** have been previously reported from the plant *Arum italicum* (Della Greca et al., 1994) whereas the aglycone of

3 has never been isolated from natural sources. Hydroperoxy sterols belong to the large class of oxysterols that are widely occurring metabolites of terrestrial plants (Xu et al., 2013) as well as marine organisms (Sheu et al., 1997) playing crucial roles in numerous biological processes (Otaegui-Arrazola et al., 2010; Kulig et al., 2016). Oxidized sterols have been shown to be produced by either enzymatic mechanisms or by autoxidation that proceeds via free radical or non-radical pathways (Iuliano, 2011). However, in the case of allylic hydroperoxy sterols, such as **1–3**, the possibility that they are artifacts arising from autoxidation (Beckwith et al., 1989) of co-occurring **9** during drying and storage of the plant seems to be more plausible. The reaction should involve the abstraction of an allylic proton by an activated oxygen along with migration of the carbon-carbon double bond (Beckwith et al., 1989). In any case, the alternative formation as naturally sensitized photooxygenation products of β-sitosterol in the living plant cannot be ruled out for compounds **1–3**.

Finally, the antibacterial properties of selected main metabolites isolated from both extracts were evaluated against some human pathogenic Gram-positive and Gram-negative bacteria strains using the disc diffusion method. Pure compounds **11**, **13**, **15**, **16**, and **20–22** along with the inseparable mixture of **18** and **19** were tested (Table 3). Compound **11** was the most active compound against three Gram-positive bacteria. Due to the scarce amount available, new hydroperoxy sterols **1–3** were not assayed.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Jasco DIP 370 digital polarimeter. LRESI-MS were performed on a Micromass Q-TOF MicroTM coupled with a HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000MW (resolution specification 5000 FWHM, deviation <5 ppm RMS in the presence of a known lock mass). High resolution mass spectra (HRESI-MS) were acquired on a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer

(Thermo Scientific). NMR experiments were recorded at ICB-NMR Service Centre. Chemical shifts values are reported in ppm and referenced to internal signals of residual protons (C_5D_5N, ^1H δ 7.19, 7.55, 8.71, ^{13}C 123.5, 135.5, 149.9 ppm; CD_3OD, ^1H δ 3.34, ^{13}C 49.0 ppm). 1D- and 2D-NMR spectra were acquired on a Bruker Avance- 400 operating at 400 MHz, using an inverse probe fitted with a gradient along the Z-axis, a Bruker Avance III HD spectrometer equipped with a CryoProbe Prodigy operating at 400 MHz, and a Bruker DRX-600 operating at 600 MHz, using an inverse TCI CryoProbe fitted with a gradient along the Z axis. HPLC separation was performed on a Shimadzu high-performance liquid chromatography using a Shimadzu liquid chromatograph LC-10AD equipped with an UV SPD-10A wavelength detector, with a reversed-phase column (Kinetex PFP, Phenomenex, 4.6 mm i.d. x 250 mm). Silica-gel chromatography was performed using pre-coated Merck F254 plates (TLC) and Merck Kieselgel 60 powder (70-230 mesh). The spots on TLC were visualized under UV light (254 nm) and then spraying them with 10 % H_2SO_4 in water followed by heating.

3.2 Plant material

The aerial parts of *G. brunneum* were collected in Ain Touta region, Batna (Algeria) in May 2012 and identified by Prof. Bachir Oudjehih, Institute of Agronomy and Veterinary Sciences, University of Batna 1 (Algeria). A voucher specimen is deposited in the herbarium of the department of the same University with code 05/ISAV/DAG/2014.

3.3 Extraction and isolation of compounds

Dried aerial parts (1 kg) were macerated with MeOH/H_2O, 8:2 (10 L × 3), at room temperature. After filtration, the organic solvent was evaporated in vacuo to give 500 mL of aqueous phase that was extracted liquid–liquid subsequently by petroleum ether (300 mL × 3), EtOAc (300 mL × 3) and BuOH (300 mL × 3). The organic phases were evaporated to give the corresponding extracts (7.0 g, 10.0 g and 35.0 g, respectively).

An aliquot (7.0 g) of the EtOAc extract was chromatographed on a SiO_2 gel column using hexane/EtOAc gradient (100:0 to 0:100, v/v) and $CHCl_3$/MeOH (70:30 to 0:100, v/v), to afford 12

fractions (Fr$_{EtOAc}$A-L). Selected fractions containing compounds of interest were considered for subsequent purification steps. Fraction Fr$_{EtOAc}$C was fractionated by Sephadex LH-20 and eluted in isocratic mode with CHCl$_3$/MeOH (1:1), to give three subfractions (Fr$_{EtOAc}$C1-C3). Subfraction Fr$_{EtOAc}$C2 was subjected to C18 cartridge (SPE) and eluted with a gradient of MeOH in H$_2$O (from 0 to 100%) to afford 3 subfractions [Fr$_{EtOAc}$C-I–C-III)]. Subfraction Fr$_{EtOAc}$C-III was purified by preparative TLC using CHCl$_3$-MeOH (95/0.5) mixtures to give compounds **4** (1.8 mg), **5** (1.2 mg), and **6** (1.5 mg). Fraction Fr$_{EtOAc}$E was submitted to a SiO$_2$ gel column eluted with a CHCl$_3$/MeOH gradient to a□ord 11 subfraction (Fr$_{EtOAc}$E1-E11). Subfraction Fr$_{EtOAc}$E4 was purified on a C18 cartridge (SPE) by using a gradient of MeOH in H$_2$O to get 7 subfractions (Fr$_{EtOAc}$E4-I-E4-VII). Subfractions Fr$_{EtOAc}$E-II, Fr$_{EtOAc}$E-IV, and Fr$_{EtOAc}$E-VII were purified on Sephadex LH-20 CC eluting with CHCl$_3$/MeOH (1:1) to provide compounds **7** (3.0 mg), **8** (1.0 mg), and **9** (10.0 mg), sequentially. Subfraction Fr$_{EtOAc}$E-V was further purified by reversed-phase HPLC (C18 Kinetex PFP, 4.6 x 250 mm) with a gradient of MeOH in H$_2$O from 50% to 100% to yield compound **10** (1.0 mg, R$_t$ 29.8 min). Fraction Fr$_{EtOAc}$E5 was chromatographed on a C18 cartridge (SPE) using MeOH-H$_2$O gradient to give five subfractions (Fr$_{EtOAc}$E5-I-E5-V). Subfraction Fr$_{EtOAc}$E5-V was further purified by reversed-phase HPLC (C18 Kinetex PFP, 4.6 x 250 mm) with a MeOH in H$_2$O gradient (from 95% to 100% in 40 min) to yield hydroperoxy sterols **1** (0.8 mg, R$_t$ 11.9 min), **2** (2.0 mg, R$_t$ 15.2 min), and **3** (0.5 mg, R$_t$ 17.8 min). Subfracion Fr$_{EtOAc}$E11 was purified on a C18 cartridge (SPE) by using a MeOH/H$_2$O gradient to get compounds **11** (11.0 mg) and **12** (4.0 mg). Fraction Fr$_{EtOAc}$G was fractionated on VLC C8 eluted with MeOH/ H$_2$O gradient to give 7 subfractions (Fr$_{EtOAc}$G1-G7). Subfraction Fr$_{EtOAc}$G1 was subjected to CC on C18 cartridge (SPE) using MeOH/H$_2$O gradient and following purification on CC Sephadex LH-20 (CHCl$_3$/MeOH, 1:1) to give compounds **13** (3.0 mg) and **14** (8.0 mg). A third of the BuOH extract (10 g) was chromatographed on a SiO$_2$ gel column using a gradient of MeOH in CHCl$_3$ (from 0 to 100 %) to afford 13 fractions (F$_{BuOH}$A-M). Selected fractions containing compounds of interest were considered for further purification steps. Fraction Fr$_{BuOH}$D was further purified on a SiO$_2$ gel column using the same conditions as above to give 7

subfractions (Fr$_{BuOH}$D1-D7). Subfraction Fr$_{BuOH}$D2 and subfraction Fr$_{BuOH}$D5 were purified by Sephadex LH-20 and eluted in isocratic mode with CHCl$_3$-MeOH (1/1) to give compounds **15** (40.6 mg) and **16** (47.0 mg), respectively. Fraction Fr$_{BuOH}$E was subjected to open CC on normal phase silica gel using CHCl$_3$/MeOH gradient as eluent. Eight fractions were collected (Fr$_{BuOH}$E1-E8). Subfraction Fr$_{BuOH}$E5 was purified by Sephadex LH-20 (CHCl$_3$/MeOH, 1:1) to yield compound **17** (4.0 mg). Subfraction Fr$_{BuOH}$E7 was fractionated on C18 cartridge (SPE) eluted with a gradient of MeOH in H$_2$O (from 0 to 10%) to give an inseparable mixture (6.0 mg) constituted by **18** and **19**. Selected fractions Fr$_{BuOH}$I and Fr$_{BuOH}$L were combined and subjected to SiO$_2$ gel column (CHCl$_3$/MeOH 99: 0.1 to 90:10) and Sephadex LH-20 column (CHCl$_3$/MeOH, 1:1), sequentially, obtaining twelve subfractions (Fr$_{BuOH}$IL1-IL12). Subfractions Fr$_{BuOH}$IL6, Fr$_{BuOH}$IL7, and Fr$_{BuOH}$IL8 contained pure compounds **20** (40 mg), **21** (32 mg), and **22** (16.0 mg), respectively. Finally, selected subfracions Fr$_{BuOH}$IL9-12 were further purified by C18 SPE cartridge using a MeOH/H$_2$O gradient to give compounds **23** (8.0 mg), **24** (2.0 mg), and **25** (4.0 mg).

3.4 Spectroscopic data

(24*R*) 24-ethyl-7α-hydroperoxy-cholest-5-en-3-*O*-β-D-glucopyranoside (**1**): White powder; [α]$^{25}_D$ − 23.5 (c 0.05, MeOH); ^{1}H- and ^{13}C-NMR (Pyr-d_5) see Table 1 and 2; ^{1}H-NMR spectral data (in MeOD, 600 MHz) δ: 5.79 (1H, br dd, *J*= 4.9, 1.3 Hz, H-6), 4.43 (1H, d, *J*= 7.9 Hz, H-1'), 4.08 (1H, dd, *J*= 4.9, 4.0 Hz, H-7), 3.91 (1H, m, H-6'a), 3.70 (1H, m, H-3), 3.69 (1H, m, H-6'b), 3.37 (1H, m, H-3'), 3.30 (1H, m, H-5'), 3.28 (1H, m, H-4'), 3.18 (1H, m, H-2'), 2.54 (1H, dd, *J* = 13.0, 4.5 Hz, H-4a), 2.37 (1H, dd, *J* = 13.0, 11.0 Hz, H-4b), 2.15 (1H, m, H-15a), 2.04 (1H, m, H-12a), 1.96 (1H, m, H-23a), 1.93 (1H, m, H-2a), 1.89 (2H, m, H$_2$-1), 1.72 (1H, m, H-25), 1.63 (1H, ddd, *J* = 11.7, 11.7, 4.0 Hz, H-8), 1.53 (2H, m, H$_2$-11), 1.51 (1H, m, H-14), 1.42 (1H, m, H-20), 1.41 (1H, m, H-9), 1.40 (1H, m, H-22a), 1.32 (4H, m, H$_2$-15 and H$_2$-28), 1.30 (1H, m, H-2b), 1.21 (2H, m, H$_2$-16), 1.20 (1H, m, H-22b), 1.19 (1H, m, H-17), 1.18 (1H, m, H-12b), 1.15 (1H, m, H-23b), 1.04 (3H, s, H$_3$-19), 0.99 (3H, d, *J* = 6.5 Hz, H$_3$-21), 0.97 (1H, m, H-24), 0.73 (3H, s, H$_3$-18), 0.88 (3H, d, *J* = 7.0 Hz, H$_3$-27), 0.87 (3H, d, *J* = 7.0 Hz, H$_3$-26), 0.90 (3H, t, *J* = 7.5 Hz, H$_3$-29); ^{13}C-NMR spectral data (in

MeOD, 100 MHz) δ: 148.4 (C, C-5), 122.4 (CH, C-6), 102.5 (CH, C-1'), 79.4 (CH, C-3), 79.2 (CH, C-7), 78.1 (CH, C-3'), 77.9 (CH, C-5'), 75.1 (CH, C-2'), 71.7 (CH, C-4'), 62.8 (CH_2, C-6'), 57.2 (CH, C-17), 50.3 (CH, C-14), 47.3 (CH, C-24), 44.9 (CH, C-9), 43.4 (C, C-13), 40.7 (CH_2, C-12), 39.8 (CH_2, C-4), 38.4 (CH, C-8), 38.2 (CH_2, C-1), 37.8 (C, C-10), 37.5 (CH, C-20), 35.1 (CH_2, C-22), 30.4 (CH, C-25), 29.4 (CH_2, C-2), 27.2 (CH_2, C-16), 25.3 (CH_2, C-23), 24.2 (CH_2, C-15 and C-28), 22.0 (CH_2, C-11), 20.2 (CH_3, C-26), 19.41 (CH_3, C-27), 19.36 (CH_3, C-21), 18.6 (CH_3, C-19), 12.3 (CH_3, C-29), 11.7 (CH_3, C-18); ESI-MS (pos. ion mode) m/z 631 $[M+Na]^+$; HR-ESI-MS m/z 631.4191 $[M+Na]^+$ (calcd for $C_{35}H_{60}O_8Na$ 631.4180).

(24R) 24-ethyl-5α-hydroperoxy-cholest-6-en-3-O-β-D-glucopyranoside (**2**): White powder; $[\alpha]^{25}_D$ − 9.2 (c 0.2, MeOH); ^{1}H- and ^{13}C-NMR (Pyr-d_5) see Table 1 and 2; ^{1}H-NMR spectral data (in MeOD, 600 MHz) δ: 5.78 (1H, dd, J= 10.0, 1.8 Hz, H-7), 5.64 (1H, dd, J= 10.0, 2.7 Hz, H-6), 4.44 (1H, d, J= 7.9 Hz, H-1'), 4.18 (1H, m, H-3), 3.90 (1H, br d, J = 11.0 Hz, H-6'a), 3.70 (1H, dd, J = 11.0, 4.0 Hz, , H-6'b), 3.39 (1H, m, H-3'), 3.31 (2H, m, H-4' and H-5'), 3.17 (1H, m, H-2'), 2.52 (1H, dd, J= 13.0, 5.0, H-4a), 2.07 (1H, m, H-12a), 1.99 (1H, m, H-8), 1.98 (1H, m, H-2a), 1.95 (2H, m, H_2-16), 1.82 (1H, m, H-9), 1.75 (4H, m, H_2-15), 1.71 (1H, m, H-2b), 1.70 (1H, m, H-25), 1.57 (1H, m, H-1a), 1.50 (1H, m, H-4b), 1.42 (2H, m, H-20 and H-22a), 1.39 (2H, m, H_2-11), 1.37 (1H, m, H-1b), 1.32 (2H m, H_2-28), 1.31 (1H, m, H-14), 1.24 (2H, m, H_2-23), 1.21 (1H, m, H-12b), 1.19 (1H, m, H-17), 1.08 (2H, m, H-22b), 1.00 (3H, s, H_3-19), 0.97 (3H, d, J = 6.5 Hz, H_3-21), 0.96 (1H, m, H-24), 0.90 (3H, t, J = 7.5 Hz, H_3-29), 0.88 (3H, d, J = 7.0 Hz, H_3-26), 0.86 (3H, d, J = 7.0 Hz, H_3-27), 0.76 (3H, s, H_3-18); ^{13}C-NMR spectral data (in MeOD, indirect detection from HSQCed and HMBC) δ: 135.0 (CH, C-7), 131.6 (CH, C-6), 102.9 (CH, C-1'), 84.0 (C, C-5), 78.2 (2 x CH_2, C-3' and C-5'), 75.8 (CH, C-3), 75.3 (CH, C-2'), 71.7 (CH, C-4'), 62.8 (CH_2, C-6'), 57.5 (CH, C-17), 55.1 (CH, C-14), 47.5 (CH, C-24), 45.5 (CH, C-9), 44.3 (C, C-13), 41.4 (CH_2, C-12), 40.5 (CH, C-8), 38.6 (C, C-10), 37.3 (CH, C-20), 35.0 (CH_2, C-22), 33.4 (CH_2, C-4), 30.3 (CH, C-25), 30.0 (CH_2, C-1), 29.5 (CH_2, C-16), 29.3 (CH_2, C-2), 27.1 (CH_2, C-23), 25.0 (CH_2, C-15), 24.3 (CH_2, C-28), 22.1 (CH_2, C-11), 20.3 (CH_3, C-26), 19.8 (CH_3, C-27), 19.4 (CH_3, C-21), 15.7 (CH_3, C-19), 12.5

(CH$_3$, C-18), 12.3 (CH$_3$, C-29); ESI-MS (pos. ion mode) *m/z* 631 [M+Na]$^+$; HR-ESI-MS *m/z* 631.4186 [M+Na]$^+$ (calcd for C$_{35}$H$_{60}$O$_8$Na 631.4180).

(24*R*) 24-ethyl-6β-hydroperoxy-cholest-4-en-3-*O*-β-D-glucopyranoside (**3**): White powder; [α]$^{25}_D$ +21.8 (c 0.05, MeOH); ^{1}H- and ^{13}C-NMR (Pyr-*d$_5$*) see Table 1 and 2; ^{1}H-NMR spectral data (in MeOD, 600 MHz) δ: 5.79 (1H, br s, H-4), 4.46 (1H, d, *J*= 7.8 Hz, H-1'), 4.30 (1H, m, H-3), 4.28 (1H, dd, *J*= 3.0, 3.0 Hz, H-6), 3.89 (1H, dd, *J* = 12.0, 2.0 Hz, H-6'a), 3.69 (1H, dd, *J* = 12.0, 5.4 Hz, H-6'b), 3.39 (1H, m, H-3'), 3.32 (1H, m, H-4'), 3.30 (1H, m, H-5'), 3.20 (1H, m, H-2'), 2.16 (1H, m, H-7a), 2.07 (1H, m, H-12a), 2.05 (1H, m, H-2a), 1.90 (2H, m, H$_2$-16), 1.77 (2H, m, H$_2$-1), 1.76 (1H, m, H-2b), 1.72 (2H, m, H-8 and H-25), 1.66 (2H, m, H$_2$-15), 1.51 (2H, m, H$_2$-11), 1.43 (1H, m, H-20), 1.41 (1H, m, H-22a), 1.36 (1H, m, H-28a), 1.32 (1H, m, H-23a), 1.26 (1H, m, H-28b), 1.23 (1H, m, H-23b), 1.20 (3H, s, H$_3$-19), 1.18 (1H, m, H-12b), 1.16 (1H, m, H-17), 1.10 (1H, m, H-7b), 1.06 (1H, m, H-22b), 1.02 (1H, m, H-14), 0.98 (1H, m, H-24), 0.97 (3H, d, *J* = 6.6, H$_3$-21), 0.90 (3H, t, *J* = 7.4, H$_3$-29), 0.89 (3H, d, *J* = 6.9, H$_3$-27), 0.87 (3H, d, *J* = 6.9, H$_3$-26), 0.79 (1H, m, H-9), 0.76 (3H, s, H$_3$-18); ^{13}C-NMR spectral data (in MeOD, indirect detection from HSQCed and HMBC) δ: 144.9 (C, C-5), 129.7 (CH, C-4), 102.6 (CH, C-1'), 87.7 (CH, C-6), 77.8 (CH, C-3'), 77.7 (CH, C-5'), 76.5 (CH, C-3), 74.8 (CH, C-2'), 71.5 (CH, C-4'), 62.8 (CH$_2$, C-6'), 57.4 (2 x CH, C-14 and C-17), 55.6 (CH, C-9), 47.3 (CH, C-24), 41.4 (C, C-13), 41.0 (CH$_2$, C-12), 37.8 (C, C-10), 37.7 (CH$_2$, C-1), 36.5 (CH$_2$, C-7), 37.2 (CH, C-20), 34.4 (CH$_2$, C-22), 30.6 (CH, C-8), 30.4 (CH, C-25), 28.9 (CH$_2$, C-16), 27.8 (CH$_2$, C-2), 26.4 (CH$_2$, C-23), 25.0 (CH$_2$, C-15), 23.8 (CH$_2$, C-28), 21.6 (CH$_2$, C-11), 20.3 (CH$_3$, C-19), 19.7 (2 x CH$_3$, C-26 and C-27), 19.0 (CH$_3$, C-21), 12.2 (2 x CH$_3$, C-18 and C-29); ESI-MS (pos. ion mode) *m/z* 631 [M+Na]$^+$; HR-ESI-MS *m/z* 631.4191 [M+Na]$^+$ (calcd for C$_{35}$H$_{60}$O$_8$Na 631.4180).

3.5 Hydrolysis of β-sitosterol-3-β-O-D-glucoside (**9**).

Compound **9** (2.5 mg) was dissolved in 1 ml of 1N HCl in MeOH, and the obtained solution was stirred for 12 h at 60°C. After the usual workup, the reaction mixture was dried and partitioned between CHCl$_3$ and H$_2$O. The organic and aqueous layers were separately dried and subjected to

NMR analysis. The proton spectrum of the organic part indicated to contain 3-β-sitosterol, whereas the aqueous fraction showed to be a mixture of. Benzoyl chloride (0.5 ml) was added to a dry pyridine solution (1 ml) of the α- and β-methylglucopyranosyl mixture, and the reaction was stirred for 12h at room temperature. After removal of the solvent under reduced pressure, the usual workup afforded α– and β–methyl-tetra-benzoyl-glucopyranoses in ratio of 2:1. The mixture was purified on a Pasteur pipette silica gel column (light petroleum ether/diethyl ether gradient) to give 0.8 mg and 0.4 mg of pure α– and β–tetra-benzoate which were identified by comparison with literature data (Gavagnin et al., 2007).

3.6 Antimicrobial activity

Six strains of bacteria were used as test microorganisms. The bacterial strains included Gram-positive Staphylococcus aureus ATCC 25923, *Staphylococcus aureus* ATCC 43300 and *Bacillus cereus* ATCC 11778 and Gram-negative *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Klebsiella pneunomoniae* ATCC 70603. The modified agar diffusion method was performed as described by Hossain et *al.* (Hossain et al., 2012) with some modifications. A standardized bacterial suspension was adjusted to a density of 1.0×10^7 UFC mL^{-1} from an overnight culture and poured into sterile petri dishes containing Mueller Hinton agar (MHA) using a cotton swab. EtOAc and BuOH extracts as well as pure compounds were dissolved in DMSO at a concentration of 500 μg/mL and sterilized by filtration through 0.22 μm sterile Millipore filters; then 10 μl of these solutions were spotted onto sterile filter paper discs (6 mm in diameter,Whatman no 1) and deposited in the center of the petri dish. After 24 h of incubation at 37 °C, the inhibition zones were measured in mm. Streptomycin 10 μg/disc was used as a positive control for the tested bacteria, and dimethyl sulfoxide (DMSO) as negative control. All experiments were performed in triplicate.

Conflict of interest

The authors have no conflict of interest to declare.

References

Abe, F., Yamauchi, T., 1988. 9-α-hydroxypinoresinol, 9-α-hydroxymedioresinol and related lignans from *Allamanda neriifolia*. Phytochemistry 27, 575–577. https://doi.org/10.1016/0031-9422(88)83144-3.

Agnihotri, V.K., El-Sohly, H.N., Shabana, I.K., Jacob, M.R., Joshi, V.C., Smillie, T., Khan, I.A., Walker, L.A., 2008. Constituents of *Nelumbo nucifera* leaves and their antimalarial and antifungal activity. Phytochemistry Lett. 1, 89–93. https://doi.org/10.1016/j.phytol.2008.03.003.

Badawi, M.M., Handa, S.S., Kinghorn, A.D., Cordell, G.A., Farnsworth, N.R., 1985. Plant anticancer agents XXVII: antileukemic and cytotoxic constituents of *Dirca occidentalis* (Thymelaeaceae). J. Pharm. Sci. 72, 1285–1287. https://doi.org/10.1002/jps.2600721112.

Beckwith, A.L.J., Davies, A.G., Davison, I.G.E., Maccoll, A., Mruzek, M.H., 1989. The mechanisms of the rearrangements of allylic hydroperoxides: 5α-hydroperoxy-3β-hydroxycholest-6-ene and 7α- hydroperoxy-3β-hydroxycholest-5-ene. J. Chem. Soc. Perkin Trans 2, 815–823. https://doi.org/10.1039/P29890000815.

Bitam, F., Ciavatta, M.L., Manzo, E., Dibi, A., Gavagnin, M., 2008. First chemical characterisation of the terpenoid constituents of the Algerian plant *Launaea arborescens*. Phytochemistry 69, 2984–2992. https://doi.org/10.1016/j.phytochem.2008.09.025.

Bolivar, P., Cruz-Paredes, C., Hernández, L.R., Juárez, Z.N., Sánchez-Arreola, E., Av-Gay, Y., Bach, H., 2011. Antimicrobial, anti-inflammatory, antiparasitic, and cytotoxic activities of *Galium mexicanum*. J. Ethnopharmacol. 137, 141–147. https://doi.org/10.1016/j.jep.2011.04.069.

Boumaraf, M., Carbone, M., Ciavatta, M.L., Benyahia, S., Ameddah, S., Menad, A., Benayache, S., Benayache, F., Gavagnin, M., 2017. Exploring the bioactive terpenoid content of an Algerian plant of genus *Pulicaria*: the *ent*-series of asteriscunolides. J. Nat. Prod. 80, 82–89. https://doi.org/10.1021/acs.jnatprod.6b00717.

Bouzergoune, F., Ciavatta, M.L., Bitam, F., Carbone, M., Aberkane, M.C., Gavagnin, M., 2016. Phytochemical study of *Eryngium triquetrum*: isolation of polyacetylenes and lignans. Planta Med. 82, 1438–1445. https://doi.org/10.1055/s-0042-110316.

Bradic, J., Petkovic, A., Tomovic, M., 2018. Phytochemical and pharmacological properties of some species of the genus *Galium* L. (*Galium verum* and *mollugo*). Ser. J. Exp. Clin. Res.; and references herein cited. https://doi.org/10.1515/sjecr-2017-0057.

Bremer, B., 2009. A review of molecular phylogenetic studies of Rubiaceae. Ann. Mo. Bot. Gard. 96, 4–26. https://doi.org/10.3417/2006197.

Camero, M.C, Germanò, M.P., Rapisarda, A., D'Angelo, V., Amira, S., Benchikh, F., Braca, A., De Leo, M., 2018. Anti-angiogenic activity of iridoids from *Galium tunetanum*. Braz. J. Pharmacog. 28, 374–377. https://doi.org/10.1016/j.bjp.2018.03.010.

Chaher, N., Krisa, S., Delaunay, J.-C., Bernillon, S., Pedrot, E., Mérillon, J.-M., Atmani, D., Richard, T., 2016. Unusual compounds from *Galium mollugo* and their inhibitory activities against ROS generation in human fibroblasts. J. Pharm. Biomed. Anal. 117, 79–84. https://doi.org/10.1016/j.jpba.2015.07.027.

Chaurasia, N., Wichti, M., 1987. Sterols and steryl glycosides from *Urtica dioica*. J. Nat. Prod. 50, 881–885. https://doi.org/10.1021/np50053a018.

Chen, C., Qiang, S., Lou, L., Zhao, W., 2009. Cucurbitane-type triterpenoids from the stems of *Cucumis melo*. J. Nat. Prod. 72, 824–829. https://doi.org/10.1021/np800692t.

Cheng, D.-L., Cao, X.-P., 1992. Pomolic acid derivatives from the root of *Sanguisorba officinalis*. Phytochemistry 31, 1317–1320. https://doi.org/10.1016/0031-9422(92)80499-5.

Chinese Materia Medica, 1977. Jiangsu New Medical College (Ed.), Vol. 2449. Shanghai People's Publishing House: Shanghai.

Della Greca, M., Fiorentino, A., Molinaro, A., Monaco, P., Previtera, L., 1994. Hydroperoxysterols in *Arum italicum*. Nat. Prod. Lett. 5, 7–14. https://doi.org/10.1080/10575639408043928.

Demirezer, L.O., Gürbüz, F., Güvenalp, Z., Str☐ch, K., Zeeck, A., 2006. Iridoids, flavonoids and monoterpene glycosides from *Galium verum* subsp. verum. Turk. J. Chem. 30, 525–534.

Dickey, E.E., 1958. Liriodendrin, a new lignan diglucoside from the inner bark of yellow poplar (*Liriodendron tulipifera* L.). J. Org. Chem. 23, 179–184. https://doi.org/10.1021/jo01096a007.

Djebara, A., Ciavatta, M.L., Mathieu, V., Margaux, C., Bitam, F., Carbone, M., Gavagnin, M., 2019. Oxygenated C17 polyacetylene metabolites from Algerian *Eryngium tricuspidatum* L. roots: structure and biological activity. Fitoterapia 138, e104355. https://doi.org/10.1016/j.fitote.2019.104355.

Ehrendorfer, F., Barfuss, M.H.J., Manen, J.-F., Schneeweiss, G.M., 2018. Phylogeny, character evolution and spatiotemporal diversification of the species-rich and world-wide distributed tribe Rubieae (Rubiaceae). PLoS One 13, e0207615. https://doi.org/10.1371/journal.pone.0207615.

Ehrendorfer, F., Krendl, F., Puff, C., 1976. *Galium* L. In: Tutin, T. (Ed.), Flora Europaea, Cambridge University Press, Cambridge, pp. 14–36.

El-Gamal, A.A., Takeya, K., Itokawa, H., Halim, A.F., Amer, M.M., Saad, H.-E.A., 1997. Lignan bis-glucosides from *Galium sinaicum*. Phytochemistry 45, 597–600. https://doi.org/10.1016/S0031-9422(96)00881-3.

El-Naggar, L.J., Beal, J.L., 1980. Iridoids: a review. J. Nat. Prod. 43, 649–707. https://doi.org/10.1021/np50012a001.

Gaamoune, S., Harzallah, D., Kada, S., Dahamna, S., 2014. Evaluation of antioxidant activity of flavonoids extracted from *Galium tunetanum* Poiret. Res. J. Pharm. Biol. Chem. Sci. 5, 341–348.

Gavagnin, M., Carbone, M., Amodeo, P., Mollo, E., Vitale, R.M., Roussis, V., Cimino, G., 2007. Structure and absolute stereochemistry of syphonoside, a unique macrocyclic glycoterpenoid from marine organism. J. Org. Chem. 72, 5625–5630. https://doi.org/10.1021/jo0704917.

Haruna, M., Koube, T., Ito, K., Murata, H., 1982. Balanophonin, a new neo-lignan from *Balanophora japonica* Makino. Chem. Pharm. Bull. 30, 1525–1527. https://doi.org/10.1248/cpb.30.1525.

Holland, H.L., Jahangir, 1983. Reactions of steroidal 4,5- and 5,6-epoxides with strong bases. Can. J. Chem. 61, 2165–2170. https://doi.org/10.1139/v83-377.

Hossain, M.A., Shah, M.D., Sang, S.V., Sakari, M., 2012. Chemical composition and antibacterial properties of the essential oils and crude extracts of *Merremia borneensis.* J. King Saud. Univ. 24, 243–249. https://doi.org/10.1016/j.jksus.2011.03.006.

Iuliano, L., 2011. Pathways of cholesterol oxidation via non-enzymatic mechanisms. Chem. Phys. Lipids 164, 457–468. https://doi.org/10.1016/j.chemphyslip.2011.06.006.

Jarić, S., Popović, Z., Mačukanović-Jocić, M., Djurdjević, L., Mijatović, M., Karadžić, B., Mitrović, M., Pavlović, P., 2007. An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). J. Ethnopharmacol. 111, 160–175. https://doi.org/10.1016/j.jep.2006.11.007.

Kaval, I., Behçet, L., Cakilcioglu, U., 2014. Ethnobotanical study on medicinal plants in Geçitli and its surrounding (Hakkari-Turkey). J. Ethnopharmacol. 155, 171–184. https://doi.org/10.1016/j.jep.2014.05.014.

Kulig, W., Cwiklik, L., Jurkiewicz, P., Rog, T., Vattulainen, I., 2016. Cholesterol oxidation products and their biological importance. Chem. Phys. Lipids 199, 144–160. https://doi.org/10.1016/j.chemphyslip.2016.03.001.

Mabry, T.J., Markham, K.R., Thomas, M.B., 1970. The systematic identification of flavonoids, Springer-Verlag, Berlin.

Martins, D., Nunez, C.V., 2015. Secondary metabolites from Rubiaceae species. Molecules 20, 13422–13495; and references herein cited. https://doi.org/10.3390/molecules200713422.

Mocan, A., Cri□an, G., Vlase, L., Ivănescu, B., Bădărău, A.S., Arsene, A.L., 2016. Phytochemical investigations on four *Galium* species (Rubiaceae) from Romania. Farmacia 64, 95–98; and references herein cited.

Nagai, T., Kiyohara, H., Munakata, K., Shirahata, T., Sunazuka, T., Harigaya, Y., Yamada, H., 2002. Pinellic acid from the tuber of *Pinellia ternata* Breitenbach as an effective oral adjuvant for nasal influenza vaccine. Int. Immunopharm. 2, 1183–1193. https://doi.org/10.1016/s1567-5769(02)00086-3.

Ogawa, K., Kawasaki, A., Omura, M., Yoshida, T., Ikoma, Y., Masamichi, Y., 2001. 3',5'-Di-*C*-β-glucopyranosylphloretin, a flavonoid characteristic of the genus *Fortunella*. Phytochemistry 57, 737–742. https://doi.org/10.1016/s0031-9422(01)00132-7.

Otaegui-Arrazola, A., Menéndez-Carreño, M., Ansorena, D., Astiasarán, I., 2010. Oxysterols: a world to explore. Food Chem. Toxicol. 48, 3289–3303. https://doi.org/10.1016/j.fct.2010.09.023.

Otsuka, H., Yoshimura, K., Yamasaki, K., Cantoria, M.C., 1991. Isolation of 10-*O*-acetyl iridoid glucosides from a Philippine medicinal plant, *Oldenlandia corymbosa* L. (Rubiaceae). Chem. Pharm. Bull. 39, 2049–2052. https://doi.org/10.1248/cpb.39.2049.

Ouyang, F., Liu, Y., Li, R., Li, L., Wang, N.-L., Yao, X.-S., 2011. Five lignans and an iridoid from *Sambucus williamsii*. Chin. J. Nat. Med. 9, 26–29. https://doi.org/10.1016/S1875-5364(11)60014-9.

Quezel, P., Santa, S., 1963. In: Nouvelle Flore de l'Algérie et des Régions Désertiques Méridionales, Vol. 1–2. CNRS, Paris. pp.650.

Robbrecht, E., 1988. Tropical woody Rubiaceae. Oper. Bot. Belg. 1, 599–602.

Rui-Lin, N., Tanaka, T., Zhou, J., Tanaka, O., 1982. Phlorizin and trilobatin, sweet dihydrochalcone-glucosides from leaves of *Lithocarpus litseifolius* (Hance) Rehd. (Fagaceae). Agric. Biol. Chem. 46, 1933–1934. https://doi.org/10.1080/00021369.1982.10865357.

Sakakibara, J., Kaiya, T., Fukuda, H., Ohki, T., 1983. 6β-Hydroxyursolic acid and other triterpenboids of *Enkianthus cernuus*. Phytochemistry 22, 2553–2555. https://doi.org/10.1016/0031-9422(83)80162-9.

Salama, O., Chaudhuri, R.K., Sticher, O., 1981. A lignan glucoside from *Euphrasia rostkoviana*. Phytochemistry 20, 2603–2604. https://doi.org/10.1016/0031-9422(81)83110-X.

Shah, S.R.U., Quasim, M., Khan, I.A., Shah, S.A.U., 2006. Study of medicinal plants among weeds of wheat and maize in Peshawar region. Pak. J. Weed Sci. Res. 12, 191–197.

Sheu, J.-H., Wang, G.-H., Sung, P.-J., Chiu, Y.-H., Duh, C.-Y., 1997. Cytotoxic sterols from the Formosan brown alga *Turbinaria ornata*. Planta Med. 63, 571–572. https://doi.org/10.1055/s-2006-957772.

Shi, D., Fan, X., Sun, J., Han, L., Shi, J., 2008. Steroids from green alga *Chaetomorpha basiretorsa* Setchell. Chin. J. Oceanol. Limn. 26, 415–418. https://doi.org/10.1007/s00343-008-0415-7.

Smadi, A., 2018. Etude phytochimique des plantes *Cymodocea nodosa* et *Galium brunneum*. PhD thesis, University of Batna 1.

Stuppner, H., Wagner, H., 1989. New cucurbitacin glycosides from *Picrorhiza kurrooa*. Planta Med. 55, 559–563. https://doi.org/10.1055/s-2006-962095.

Sy, L.-K., Brown, G.D., 1999. Coniferaldehyde derivatives from tissue culture of *Artemisia annua* and *Tanacetum parthenium*. Phytochemistry 50, 781–785. https://doi.org/10.1016/S0031-9422(98)00607-4.

The Plant List, 2013. Version 1.1. Published on the Internet; http://www.theplantlist.org/ (accessed on 1st December 2019).

Wang, Q.-H., Peng, K., Tan, L.-H, Dai, H.-F., 2010. Aquilarin A, a new benzenoid derivative from the fresh stem of *Aquilaria sinensis*. Molecules 15, 4011–4016. https://doi.org/10.3390/molecules15064011.

Wright, J.L.C., McInnes, A.G., Shimizu, S., Smith, D.G., Walter, J.A., Idler, D., Khalil, W., 1978. Identification of C-24 alkyl epimers of marine sterols by ^{13}C nuclear magnetic resonance spectroscopy. Can. J. Chem. 56, 1898–1903. https://doi.org/10.1139/v78-308.

Xu, R.-L., Wang, R., Ding, L., Shi, Y.-P., 2013. New cytotoxic steroids from the leaves of *Clerodendrum trichotomum*. Steroids 78, 711–716. https://doi.org/10.1016/j.steroids.2013.03.002.

Yamada, Y., Hagiwara, K., Iguchi, K., Suzuki, S., 1977. Structures of arvenin I and II, bitter principles from *Anagallis arvensis* L. (Primulaceae). New cucurbitacin glucosides. Tetrahedron Lett. 18, 2099–2102. https://doi.org/10.1016/S0040-4039(01)83690-X.

Zergainoh, N., Ciavatta, M.L., Carbone, M., Bitam, F., Aberkane, M.C., Gavagnin, M., 2018. Exploring the chemical diversity of Algerian plants: three new pentacyclic triterpenoids from *Launaea acanthoclada* roots. Molecules 23, e80. https://doi.org/10.3390/molecules23010080.

Zhao, C.-C., Shao, J.-H., Li, X., Xu, J., Zhang, P., 2005. Antimicrobial constituents from fruits of *Ailanthus altissima* Swingle. Arch. Pharm. Res. 28,1147–1151. https://doi.org/10.1007/bf02972977.

Zhao, C.-C., Wang, J.H., Li, W., Sha, Y., Li, X., 2003. Studies on the chemical constituents of fructus *Ailanthi altissimae*. Chin. J. Med. Chem. 13, 211–214.
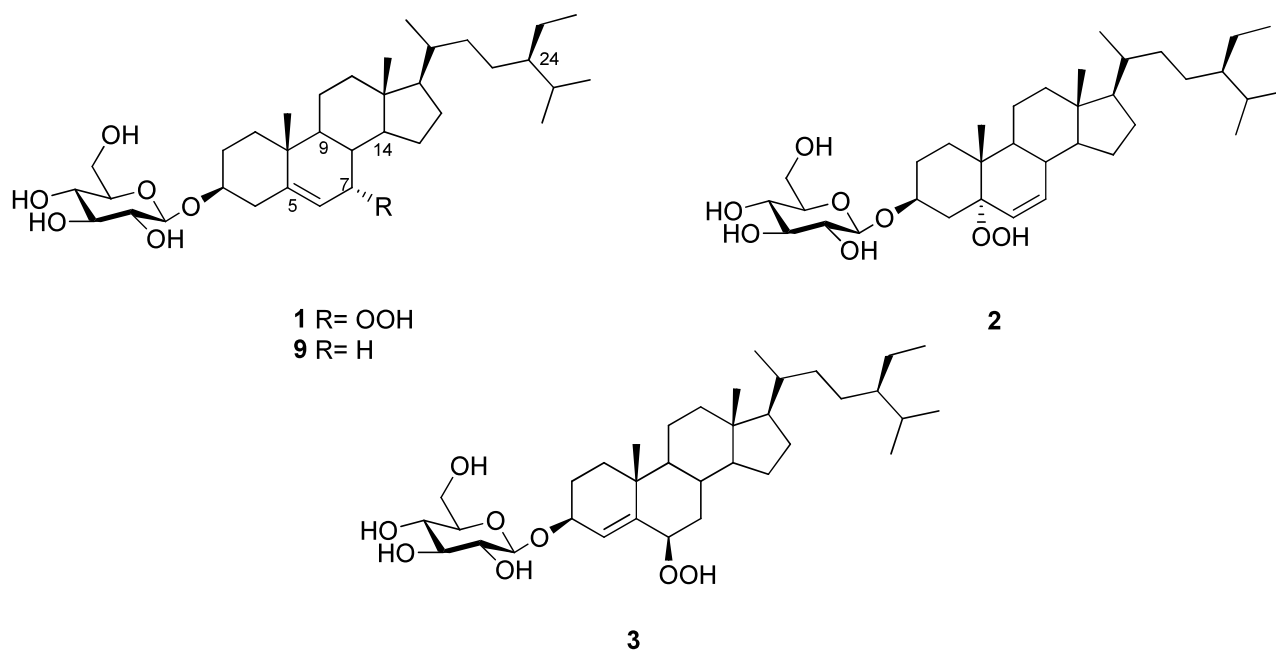
1 R= OOH
9 R= H

2

3

Fig. 1 Structures of hydroperoxy sterols **1-3** and related known sterol **9** from *G.brunneum*

Table 1. ^{1}H NMR data[a] in Pyr-d_5 for compounds **1-3** and **9**.

	1	**2**	**3**	**9**[b]
	δ_H, m (J in Hz)	δ_H, m (J in Hz)	δ_H, m (J in Hz)	δ_H, m (J in Hz)
1	1.71, m 1.07, m	1.78, m 1.30, m	1.82, m 1.02, m	1.73, m 0.99, m
2	2.08, m 1.73, m	2.30, m 1.95, m	2.16, m 1.90, m	2.14, m 1.75, m
3	3.84, m	4.85, m	4.48, m	3.93, m
4	2.77, dd (13, 4.5) 2.53, dd (13, 11.7)	3.16, dd (13, 4.5) 1.78, m	6.18, br s	2.71, br dd 13.0, 2.4 2.46, dd 13.0, 11.0
5				
6	6.07, dd (4.7, 1.5)	5.92, dd (10.0, 2.5)	4.63, m	5.34, br s
7	4.36, dd (4.0, 4.0)	5.68, dd (10.0, 1.5)	1.92, m 1.11, m	1.90, m 1.54, m
8	1.60, m	1.88, m	1.65, m	1.38, m
9	1.62, m	1.97, m	0.80, m	0.91, m
11	1.43, m	1.30, m 1.18, m	1.41, m	1.43, m
12	1.97, m 1.14, m	1.90, m 0.99, m	2.18, m 1.10, m	1.98, m 1.11, m
14	1.84, m	0.99, m	1.01, m	0.97, m
15	2.15, m 1.23, m	1.21, m	1.63, m	1.57, m 1.05, m
16	1.87, m 1.26, m	1.24, m	1.82, m	1.85, m
17	1.13, m	1.02, m	1.10, m	1.11, m
18	0.68, s	0.64, s	0.70, s	0.66, s
19	0.91, s	0.92, s	1.33, s	0.94, s
20	1.40, m	1.36, m	1.37, m	1.40, m
21	0.96, d (6.2)	0.93, d (6.2)	0.98, d (6.2)	0.99, d (6.6)
22	1.08, m	1.36, m 1.01, m	1.38, m	1.41, m 1.09, m
23	1.23, m	1.51, m 1.14, m	1.34, m	1.25, m
24	0.97, m	0.95, m	0.98, m	1.00, m
25	1.65, m	1.67, m	1.67, m	1.67, m
26	0.85, d (6.8)	0.88, d (6.8)	0.86, d (6.8)	0.86, d (6.7)
27	0.85, d (6.8)	0.85, d (6.8)	0.86, d (6.8)	0.88, d (6.8)
28	1.26, m	1.26, m	1.32, m 1.24, m	1.29, m
29	0.88, t (6.8)	0.88, t (6.8)	0.88, t (6.8)	0.89, t (7.0)
1'	4.93, d (7.7)	5.10, d (7.9)	5.11, d (7.6)	5.05, d (8.0)
2'	4.05, m	4.05, m	4.08, m	4.05, m
3'	4.28, m	4.25, m	4.30, m	4.27, m
4'	4.27, m	4.30, m	4.28, m	4.26, m
5'	3.97, m	3.82, m	4.02, m	3.98, m
6'	4.58, m 4.42, m	4.44, m	4.60, m 4.42, m	4.55, ddd (12.0, 7.0, 2.0 4.40, dd (12.0, 7.0)

[a] Assignments aided by COSY, HSQC edited, and HMBC (J = 7 Hz) experiments

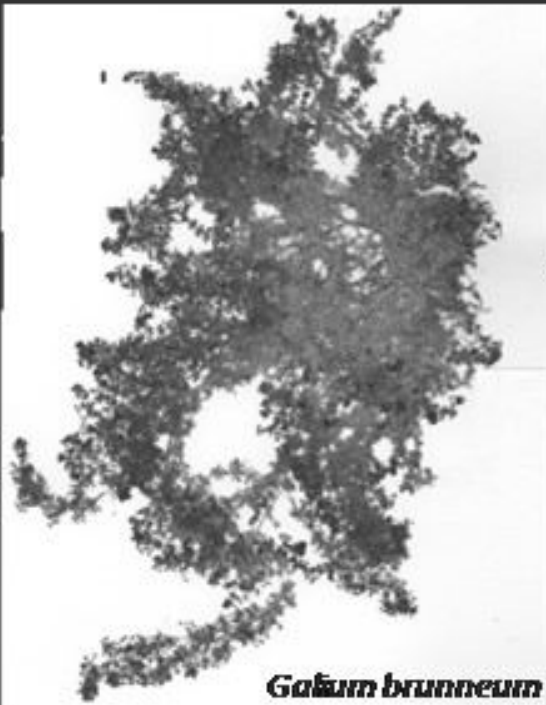[b] Assignmens in agreement with the literature

Table 2. ^{13}C NMR data^a in Pyr-d_5 for compounds **1-3** and **9**.

C	1 δ_C, m	2 δ_C, m	3 δ_C, m	9^b δ_C, m
1	37.7, CH$_2$	29.2, CH$_2$	36.9, CH$_2$	37.3, CH$_2$
2	30.1, CH$_2$	29.5, CH$_2$	27.7, CH$_2$	30.0, CH$_2$
3	78.53, CH	75.2, CH	72.9, CH	77.9, CH
4	39.5, CH$_2$	33.7, CH$_2$	129.6, CH	39.1, CH$_2$
5	146.5, C	82.8, C	143.3, C	140.9, C
6	122.7, CH	131.8, CH	75.0, CH	121.7, CH
7	77.8, CH	133.6, CH	39.9, CH$_2$	32.0, CH$_2$
8	37.4, CH	39.14, CH	30.1, CH	31.8, CH
9	43.9, CH	44.3, CH	54.6, CH	50.1, CH
10	37.8, C	38.7, C	37.0, C	37.3, C
11	20.0, CH$_2$	21.2, CH$_2$	21.3, CH$_2$	21.1, CH$_2$
12	39.5, CH$_2$	40.3, CH$_2$	40.0, CH$_2$	39.7, CH$_2$
13	42.5, C	43.7, C	42.7, C	42.3, C
14	49.4, CH	53.8, CH	56.5, CH	56.6, CH
15	24.7, CH$_2$	26.4, CH$_2$	25.0, CH$_2$	24.3, CH$_2$
16	28.6, CH$_2$	28.6, CH$_2$	28.2, CH$_2$	28.3, CH$_2$
17	56.1, CH	56.1, CH	56.3, CH	56.0, CH
18	11.5, CH$_3$	12.2, CH$_3$	11.9, CH$_3$	11.7, CH$_3$
19	24.7, CH$_3$	15.4, CH$_3$	20.0, CH$_3$	19.2, CH$_3$
20	36.4, CH	36.4, CH	36.8, CH	36.1, CH
21	18.2, CH$_3$	18.9, CH$_3$	18.9, CH$_3$	19.0, CH$_3$
22	34.2, CH$_2$	34.2, CH$_2$	34.0, CH$_2$	34.0, CH$_2$
23	26.3, CH$_2$	24.0, CH$_2$	26.9, CH$_2$	26.2, CH$_2$
24	46.1, CH	46.1, CH	46.1, CH	45.8, CH
25	29.5, CH	29.4, CH	30.0, CH	29.2, CH
26	19.1, CH$_3$	19.9, CH$_3$	19.3, CH$_3$	19.7, CH$_3$
27	19.2, CH$_3$	19.2, CH$_3$	19.3, CH$_3$	18.8, CH$_3$
28	23.4, CH$_2$	23.4, CH$_2$	23.0, CH$_2$	23.2, CH$_2$
29	12.2, CH$_3$	12.2, CH$_3$	11.8, CH$_3$	11.9, CH$_3$
1'	102.9, CH	102.9, CH	103.1, CH	102.4, CH
2'	75.3, CH	75.3, CH	75.2, CH	75.1, CH
3'	78.6, CH	78.6, CH	78.7, CH	78.4, CH
4'	71.6, CH	71.6, CH	71.9, CH	71.5, CH
5'	78.57, CH	78.5, CH	78.6, CH	78.3, CH
6'	62.9, CH$_2$	62.9, CH$_2$	62.8, CH$_2$	62.6, CH$_2$

^{a}Assignments aided by HSQC edited and HMBC (J = 7 Hz) experiments
^{b} Assignmens in agreement with the literature

Table 3. Antibacterial activity of selected compounds of *G. brunneum* by disk diffusion method

Compound	nmol/ disk	Gram+			Gram-		
		Staphylococcus aureus **ATCC 25923**	*Staphylococcus aureus* **ATCC 43300**	*Bacillus cereus* **ATCC 11778**	*Pseudomonas aeruginosa* **ATCC 27853**	*Escherichia coli* **ATCC 25922**	*Klebsiella pneunomoniae* **ATCC 70603**
11	23	9.8 ± 0.8	11.0 ± 0.5	13.8 ± 0.3	< 7	8.7 ± 0.6	7.0 ± 0.0
13	21	7.8 ± 0.8	7.0 ± 0.0	9.8 ± 0.8	< 7	8.3 ± 0.6	7.7 ± 0.6
15	19	7.7 ± 0.6	7.3 ± 0.6	10.0 ± 0.0	< 7	9.8 ± 0.8	7.7 ± 0.6
16	24	7.2 ± 0.3	7.2 ± 0.3	9.2 ± 0.3	< 7	10.0 ± 0.0	7.0 ± 0.0
18+19	19	15.2 ± 0.8	10.7 ± 0.6	10.7 ± 0.6	< 7	7.7 ± 0.6	7.7 ± 0.6
20	16	7.5 ± 0.5	7.7 ± 0.6	10.7 ± 0.6	< 7	8.7 ± 0.6	7.5 ± 0.9
21	16	10.2 ± 0.8	10.0 ± 0.0	10.0 ± 0.0	< 7	10.3 ± 1.2	7.2 ± 0.3
22	17	8.0 ± 1.0	7.7 ± 0.8	10.8 ± 0.8	< 7	8.2 ± 0.3	8.0 ± 0.0
Streptomycin	8.6	14.3 ± 05	15.3 ± 0.5	25.0 ± 1.0	21.6 ± 0.5	15.0 ± 1.0	18.0 ± 1.0

Galium brunneum