A new δ-tocotrienolic acid derivative and other constituents from the cones of *Cedrus atlantica* and their *in vitro* antimicrobial activity

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Abstract

The phytochemical and antimicrobial properties of the cones of *Cedrus atlantica* (Endl) Manetti ex Carrière were investigated. Two new compounds (**1-2**) and nineteen known compounds (**3-21**) were isolated. Their structures were established by mass spectrometry (HRESIMS), 1D, 2D NMR and by comparison with literature data. Antimicrobial activity of hydromethanolic extract against a panel of 22 bacteria and yeasts showed an interesting antimicrobial activity. All compound purified from this extract were tested against *S. aureus* by bioautography. MIC values of the most active compounds were determined using a serial dilution technique. The results showed strong antibacterial activity of the abietane diterpenes **10, 11, 14, 15, 16** and **17**. Dehydroabietic acid (**17)** was the most potent against *Enterococcus faecalis* (MIC = 15.1 = µg/mL), a multi-resistant commensal bacterium which can cause the fatal infections in humans.

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1. Introduction

Cedrus atlantica (Endl.) Manetti ex Carrière (Pinaceae) is a large and exceptionally long-lived conifer only distributed in mountain range of Morocco and Algeria (Begona et al., 2005; Sugita et al., 2004). Essential oil of *C. atlantica* has been already studied and shown that it possessed anti-inflammatory (Sugita et al., 2004), antifungal (Bouchra et al., 2013), antimicrobial (Hammer et al., 1999, Zrira et al., 2016) properties. It's also used in the treatment against hair loss (Ormerod et al., 2000). Phytochemical study of cones of *Cedrus atlantica* has previously exhibited the richness of abietane diterpenoids. Diethylether extracts contained diterpene acids bearing the abietane and pimarane skeletons (Norin et al., 1971), five oxygenated abiet-8(14)-ene derivatives were isolated from the neutral part of the *n*-hexane extract of cones (Barrero et al., 2005) and four abietatrienoid, also isolated from the *n*-hexane extract of the cones, exhibited significant antibacterial activity against Gram (+/-) bacteria (Dakir et al., 2005). More recently, abietanes diterpenes and lignans were identified in resins of *C. atlantica* (Nam et al., 2011).

Herein, as a result of our investigation on the hydromethanolic extract of the cones of *C. atlantica*, we report the isolation, structure elucidation of a new tocotrienolic acid derivative and a new *O*-acylated flavonol glycoside together with nineteen known compounds. In addition the antimicrobial activity of the hydromethanolic extract was evaluated against 22 micro-organisms and the purified compounds were evaluated against *E. faecalis* ATCC 1034, *S. aureus* CIP 53.154, *S. epidermidis*, *E. coli* CIP 54.127 and *P. aeruginosa* ATCC 9027.

2. Results and discussion

Compounds **1-21** were isolated from hydromethanolic extract of the cones of *C. atlantica* by successive chromatographic separation, including Vaccum Liquid Chromatography, flash chromatography and semi-prep HPLC. The known compounds **3-21** have been identified as: two tocotrienol derivatives, γ-tocotrienolic acid (= γ-garcinoic acid) (**3**) (Alsabil et al., 2016) δ-(E)- deoxy- amplexichromanol (**4**) (Lavaud et al., 2015), two flavonoids: daglesioside IV (**5**) (Krauze-Baranowska et al., 2013), and (+) taxifolin (**6**) (Agrawal et al., 1981), three neolignans: *ent*- cedrusin (**7**) (Agrawal et al., 1980, Kim et al., 2013), isomassonianoside B (**8**) **(**He et al., 2011, Kim et al., 2013), and (7*R*, 8*S*) dihydrodehydrodiconiferyl alcohol (**9**) (Miyase et al., 1989), and twelve abietane diterpenoids: pomiferin A (**10**) (Fraga et al., 1994,

Yang et al., 2010), 8,11,13-abietatriene-7α,18-diol or 7α-hydroxydehydroabietinol (**11**) (Barrero et al., 1992, Yang et al., 2010), 7-oxodehydroabietinol (**12**) (Tannaka et al., 1997; Gonza´lez et al., 2010), abiesadine F (**13**)**,** abiesadine L (**14**), abiesadine R (**15**), abiesadine Q (**16**) (Yang et al., 2010), dehydroabietic acid (**17**) (Cheung et al., 1993), 12-hydroxydehydroabietic acid (**18**) (Kinouchi et al., 2000), 7α,15-dihydroxydehydroabietic acid (**19**) (Prinz et al., 2002), 7-oxodehydroabietic acid (**20**) (Yang et al., 2010), and 8,11,13-abietatriene-7α-ol (**21**) (Conner et al. 1980). Among them, three abietane diterpenes (**10**-**12**) were previously isolated from the *n*-hexane extracts of the cones of *C. atlantica* (Barrero et al., 2005, Dakir et al., 2005) with dehydroabietic acid (**17**) isolated from the cone and resin of *C. atlantica* (Nam et al, 2011, Norin et al., 1971), *C. deodara* Loud (Ohmoto et al., 1989), and *C. libani* (Avicibasi et al., 1988). In addition, (+) taxifolin (**6**), cedrusin without stereochemistry (**7**) and 7α-hydroxydehydroabietinol (**11**) were isolated from *C. deodara* (Agrawal et al., 1980, Awad et al., 2015, Ohmoto et al., 1989). All other compounds (**1-5, 8-9, 13-16, 18-21**) were isolated for the first time in the *Cedrus* genus and *ent*-cedrusin (**7**) was identified in *Abies holophyla* Maxim. (Kim et al., 2013). The two known tocotrienols derivatives (**3**-**4**), previously isolated in *Garcinia amplexicaulis* (Alsabil et al., 2016, Lavaud et al., 2015), were isolated for the first time in Pinaceae family. The other known compounds (**5**-**21**) were previously isolated from the Pinaceae family, for example, the eleven abietane diterpenes (**10**-**20**) were isolated from *Abies georgei* Orr (Yang et al., 2010) and the abietane **21** from the trunk of *Abies holophyla* (Kim et al., 2016) and the bark of *Pinus monticola* Dougl. (Conner et al., 1980).

Compound **1** (Fig. 1) was isolated as a white powder with optical rotation $[\alpha]_D^{20}$ -0.8 (MeOH). The compound gave a pseudomolecular ion $[M+Na]^+$ at *m/z* 479.2766 suggesting a molecular formula of $C_{28}H_{40}O_5$ which requires 9 degrees of insaturations. The ^{1}H NMR (Table 1), exhibited signals characteristic of the presence of an aromatic proton at δ_H 6.51 (1H, s, H-7) from a pentasubstituted aromatic ring, a benzylic methyl group at δ_H 2.06 (3H, s, H-27) , a methoxyl group at δ_H 3.76 (3H, s, H-26) and four methylene protons of a chroman ring [δ_H 1.71 (1H, dd, *J*= 13.7, 6.8 Hz, H-3a), 1.79 (1H, dd, *J*= 13.7, 6.8 Hz, H-3b), and 2.73 (2H, td, *J*= 6.8, 1.5 Hz, H-4)] (Alsabil et al., 2016, Lavaud et al., 2015). The presence of a tri-isoprenyl side chain was deduced from the observation of three olefinic protons at δ_H 5.27 (1H, t, *J*= 7.6 Hz, H-11), 5.25 (1H, t, *J*= 7.4 Hz, H-15) and 6.74 (1H, t, *J*= 6.6 Hz, H-19) and three singlets of allylic methyl groups at δ_H 1.82 (3H, s, H-22), 1.62 (3H, s, H-23), and 1.60 (3H,s, H-24) (Alsabil et al., 2016, Min-Cheol et al., 2011). In addition, it's ^{1}H and ^{13}C NMR

data showed signals of an oxyquaternary methyl group at δ_H 1.27 (3H, s, H-25, δ_C 24.4) and a carbonyl group at δ_C 172.4 as in tocotrienolic acid derivatives (Alsabil et al., 2016) (Table 1). The 12,16,20-trimethyl-11,15,19-tridecatrienoic acid side chain of **1** was established by COSY correlations of H-9 (δ_H 1.56 and 1.62) with H-10 (δ_H 2.14), H-11 (δ_H 5.27) with H-10 and H-24 (δ_H 1.60), H-13 (δ_H 2.01) with H-14 (δ_H 2.10), H-15 (δ_H 5.25) with H-14 and H-23 (δ_H 1.62), H-17 (δ_H 2.10) with H-18 (δ_H 2.29), and H-19 (δ_H 6.74) with H-18 and H-22 (δ_H 1.82) (Fig. 2). In addition, ^3J HMBC correlations between H-11/C-13, H-15/C-17 and from the carbonyl group C-21 to H-22 and H-19 indicated that the carbonyl group was located at the end of the prenyl side chain (Fig. 2). The COSY correlation of H-3 with H-4 (δ_H 2.73), and the HMBC cross peaks between H-3/C-2 (δ_C 75.9), C-4a (δ_C 116.1), C-9 (δ_C 40.3) and oxyquaternary methyl C-25 (δ_C 24.4) confirmed the presence of a chroman ring and the attachment of both 12,16,20-trimethyl-11,15,19-tridecatrienoic acid side chain and oxyquaternary methyl at C-2 of chroman moiety. The location of the substituent on the aromatic ring was deduced from analysis of correlations in the HMBC spectrum. The HMBC correlations between H-4/ C-5 (δ_C 144.4) and the methoxyl H-26 (δ_H 3.76) with C-5 indicated that the methoxyl group was located at C-5. Otherwise, the aromatic proton at δ_H 6.51 was assigned as H-7 by its ^3J HMBC correlations with C-5 and C-8a (δ_C 146.2), and ^2J HMBC correlations with C-6 (δ_C 142.8) and C-8 (δ_C 122.4). The correlations of methyl group H-27 (δ_H 2.06) with C-8, C-7 and C-8a established the linkage of the methyl at C-8 and the chemical shift of C-6 the location of an hydroxyl group at C-6. In the NOESY spectrum, correlations between H-10/H-24, H-14/H-23 and H-18/H-22 and between H-11/H-13 and H-15/H-17 indicating that all the isoprenyl units has *trans* configuration (Min-Cheol et al., 2011). According to the literature, the asymmetric C-2 configuration was defined as *R* (Drotleff & Ternes, 2001). Thus the structure of compound **1** was elucidated as 11*E*-15*E*-19*E*-9-(2,8-dimethyl-5-methoxy-chroman-6-ol)-12,16,20-trimethyl-11,15,19-tridecatrienoic acid, also named 5-methoxy-δ-tocotrienolic acid.

Compound **2** (Fig. 1) was obtained as yellow powder with a molecular formula of $C_{40}H_{34}O_{14}$ deduced from its molecular ion $[M+Na]^+$ at *m/z* 761.1851 in HR-ESI-MS. The ^1H NMR spectrum showed characteristics signals of isorhamnetin moiety (Markham and Geiger, 1994; Rosch et al. 2004; Zou et al. 2007). Signals at δ_H 6.15 (1H, d, *J*=1.9 Hz) and 6.31 (1H, d, *J*=1.9 Hz) were assigned to H-6 and H-8 of the ring A, and correlated with carbons at δ_C 98.6 and δ_C 93.4, respectively on HSQC spectrum. The ring B was identified by signals at δ_H 7.85 (1H, d, *J*=1.9 Hz, H-2'), 7.60 (1H, dd, *J*=8.4, 1.9 Hz, H-6') and 6.89 (1H, d, *J*=8.4 Hz, H-5') and by the correlation on HMBC spectrum of H-2' and H-6' with C-2 (δ_C 157.5) and

between H-5' and C-1' (δ_C 121.5) (Fig. 3). In addition HMBC cross peak indicated that methoxyl group at δ_H 3.95 (δ_C 55.3) was attached to C-3' (δ_C 147.0). ^{1}H NMR spectrum showed a signal at δ_H 5.48 (1H, d, *J*= 7.9 Hz, H-1"), characteristic of an anomeric proton of a sugar moiety linked to an aromatic ring with a coupling constant (*J*=7.9 Hz) indicating a β-configuration (Mabry et al., 1970). Starting from the anomeric proton and based on the chemical shifts and correlations on COSY and HMBC spectra, this sugar was identified as a β-glucopyranose. It is attached on the hydroxyl at C-3 position of isorhamnetin as deduced from HMBC cross peak between the anomeric proton H-1' and signal at δ_C 133.7 (C-3). In the ^{1}H NMR spectrum, two sets of supplementary aromatic protons appeared between δ_H 7.4 and 7.7 together with two pairs of signals corresponding to olefinic proton at δ_H 6.66 (1H, d, *J*= 16.0 Hz, H-2'''), 7.80 (1H, d, *J*=16.0 Hz, H-3'''), 6.29 (1H, d, *J*= 16.2 Hz, H-2'''') and 7.48 (1H, d, *J*= 16.2 Hz, H-3'''') indicating the presence of two cinnamoyl groups with *E*-configuration as in compound **5** (Krauze-Baranowska et al., 2013) (Table 1). HMBC spectrum showed correlations of glucose H-3" with C-1''' (δ_C 166.9) and H-6" with C-1'''' (δ_C 166.6) (Fig. 3) indicating that the glucose was esterified by the cinnamoyl groups in positions C-3" and C-6". Thus, compound **3** was identified as isorhamnetin (3,6-*O*-di-*E*-cinnamoyl)-3-*O*-*β*-D-glucopyranoside.

Antimicrobial assays were performed on the hydromethanolic extract from the cones of *C. atlantica* against 22 micro-organisms. The results presented in Table 2 showed different degrees of activity against 22 microbial strains. The extract was active against all Gram-positive bacteria. No antimicrobial activity was found against the following 9 strains: *Escherichia coli* CIP 54.127, *Enterobacter cloacae*, *Salmonella enterica*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Candida tropicalis*, *Candida kefyr*, *Candida albicans*, and *Cryptococcus neoformans*. The Gram positive bacteria *Bacillus subtilis*, *Enterococcus faecalis* ATCC 1034, *Micrococcus luteus*, *Streptococcus pyogenes*, and the Gram negative bacteria *Shigella soneii* were the most sensitive strains to the extract with the minimum inhibitory concentration (MIC) values equal or less than 0.3 mg/mL. Among these strains, *Enterococcus faecalis* ATCC 1034 was resistant to gentamicin and vancomycin. Yeasts were less sensitive than bacteria. Given the weak antifungal activity, we focused our study only on antibacterial activity for the following steps of the work.

After purification of the compounds from the hydromethanolic extract, a bioautography assay was applied against *Staphylococcus aureus* CIP 53.154 to identify the most active. The results, given in Table 3, showed anti-staphylococcal activity for all compounds. The abietane

diterpenes **10**, **11**, **13-18**, **20** and **21** revealed a strong antibacterial activity against *Staphylococcus aureus* with a diameter of inhibition zones larger than 1.5 cm.

To measure the MIC of active compounds, a serial dilution method was used against three Gram-positive *(Enterococcus faecalis* ATCC 1034, *Staphylococcus aureus* CIP 53.154 and *Staphylococcus epidermidis*) and two Gram-negative (*Escherichia coli* CIP 54.127 and *Pseudomonas aeruginosa* ATCC 9027) bacteria. The MICs ranged from 15.1 µg/mL to the limit concentration of 500 µg/mL. At a glance, compounds with inhibition zones larger than 1.5 cm in bioautography, clearly display lower MIC values than the others. Compound **17** was considered to be the most active compound with the lowest MIC value (15.1 µg/mL) against *E. faecalis* ATCC 1034 (resistant to both antibiotics tested), a multi-resistant commensal bacterium which can cause the fatal infections in humans. The results present this strain as the most sensible among the five bacteria tested. Abietane diterpenes **10**, **11**, **14**, **15** and **16** showed more antibacterial activity compared to the other compounds with MICs ranging from 31.2 to 250 µg/mL. From which, abiesadin R (**15**) was the most active compound against *S. epidermidis* (MIC =31.2 µg/mL) and *P. aeruginosa* ATCC 9027 (MIC =62.5 µg/mL). Comparing the activity of compound **17** with those of compounds **18** and **20**, the presence of a supplementary hydroxyl group on the aromatic ring in C-12 position (**18**) induce a more important decrease in the antibacterial activity than a ketone group in C-6 position (**20**). The tocotrienolic acid derivatives **1** and **3** were moderately active against the five tested bacteria with MICs ranging from 62 to 500 µg/mL. This activity is more important against Gram-positive bacteria. According to the table 3, in twenty one cases, a strong antibacterial activity (MIC = 15.1 to 62.5 µg/mL) was achieved with an activity very close to MICs of antibiotics. In this study all compounds with the MICs >125 µg/mL, were considered as inactive against the strain tested.

3. Conclusion

The phytochemical investigation of the hydromethanolic extract of the cones of *Cedrus atlantica* growing in the eastern Algerian, allows us to isolated two new (**1-2**) and nineteen known compounds (**3-21**). The compounds were identified as three tocotrienols, the new 5-methoxy-δ-tocotrienolic acid (**1**) and two knowns (**3-4**)**,** three flavonoids**,** the new isorhamnetin (3,6-*O*-di-*E*-cinnamoyl)-3-*O*-*β*-D-glucopyranoside (**2**), and two knowns (**5-6**), three neolignans (**7-9**), and twelve abietane diterpenes (**10-21**). To our knowledge, this is the first isolation tocotrienol derivatives in the Pinaceae family. The isolation of abietane diterpenes was characteristic of the Pinaceae family. The hydromethanolic extract showed potential antibacterial activity due to these abietane diterpenes. From these compounds

dehydroabietic acid **17** was the most active with MIC value of 15.1 and 31.2 µg/mL against *E. faecalis* and *S. aureus*, respectively, similar to antibiotic used as control.

4. Experimental

4.1 General experimental procedures

Optical rotations were determined in MeOH with a Perkin-Elmer 341 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX III 500 NMR spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz) and Bruker Avance DRX 600 NMR spectrometer (^{1}H at 600MHz and ^{13}C at 150 MHz). 2D-NMR experiments were performed using standard Bruker-micrograms. Coupling constants were expressed in Hz, and chemical shifts were given on a δ (ppm) scale. TLC was carried out on precoated silica gel 60 F_{254} (Merck), and spots were observed under UV light at 254 and 365 nm or visualized by heating after spraying with H_2SO_4 50%. Flash chromatography was performed on a C_{18} reversed phase (Reveleris C_{18} reversed phase 40g; CV 45mL/min; 45mg- 1.35g sample) and silica gel 60 (Reveleris silica 40µm, 12g; CV 17-36 mL/min; 45mg- 2.4g sample) columns. HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, an Ultimate 3000 pump, a diode array detector UVD 240S and Chromeleon software. C_{18} reversed phase column (interchrom 250x10 mm, solanum 5µm) was used for semi-preparative HPLC with a binary gradient eluent (H_2O (pH 2.4 with TFA); CH_3CN) and a flow rate of 4 mL/ min; the chromatogram was monitored at 205, 225, 254 and 350 nm.

4.2 Plant material

Cones of *Cedrus libanotica* subsp. *atlantica* (Endl.) Jahand. & Maire, synonym of *Cedrus atlantica* (Endl.) Manetti ex Carrière were collected at Hamma-Knif, Khenchla provence (Eastern Algerian) in November 2013. A voucher specimen (LOST Ca11.13) has been deposited in the herbarium of the laboratory LOST, Freres Mentouri University, Constantine, Algeria.

4.3 Extraction and isolation

The powder of air-dried cones of *C. atlantica* (1kg) was extracted with 80% MeOH (3x100mL) to give 32 g of a crude extract after evaporation of the solvent. An aliquot (20 g) of this extract was submitted to *vacuum*-liquid chromatography (VLC) on reversed-phase RP-18 silica gel with MeOH/H_2O (2:8, 4:6, 6:4, 8:2, 10:0, 400 mL each), affording 5 fractions.

Fraction 2 (4:6) (820 mg) was eluted with CH_3CN-H_20 (1:9-4:6) on RP-18 flash chromatography affording 7 sub-fractions. Sub-fractions 3 (73 mg) was purified by semi-prep HPLC on RP-18, eluted with a gradient of CH_3CN-H_20 (18:82-30:70) during 25 min yielding compounds **6** (1.3 mg, Rt= 15.207 min), **7** (3 mg, Rt= 12.861 min) and **8** (1.1 mg, Rt= 8.641 min) . Fraction 3 (6:4) (600mg) was submitted to a RP-18 flash chromatography eluted with MeOH-H_2O (25:75-6:4) to give 8 sub-fractions. Sub-fraction 5 (64.8 mg) was purified by semi-prep HPLC with CH_3CN-H_2O (3:7) during 20 min to provide compound **9** (0.9 mg, Rt= 5.98 min). Fraction 4 (8:2) (1.8 g) was fractioned by silica flash chromatography, eluted with a gradient of solvents $CHCl_3$-MeOH-H_2O (10:0:0 - 0:0:10) affording 11 sub-fractions. Sub-fraction 2 (180 mg) was purified by flash chromatography on RP-18 with a gradient of solvent CH_3CN-H_2O (40:6-10:0) to afford 8 sub-fractions, purified by semi-prep HPLC on RP-18, the third one was eluted by CH_3CN-H_2O (55:45) during 16 min to give compound **18** (3.1g, Rt= 13.83 min) and the sixth sub-fraction was eluted by CH_3CN-H_2O (65:35) during 20 min yielding compounds **20** (2.4 mg, Rt= 12.24 min), **16** (2.1 mg, Rt= 13.11 min), and **12** (2.3 mg, Rt= 14.27 min). Sub-fractions 5 was purified by silica gel flash chromatography with a gradient of Hexane-AcOEt-MeOH (10:0:0 – 0:0:10) the resulting sub-fraction was purified by semi-prep HPLC on RP-18 using a gradient of solvents CH_3CN-H_2O (55:45) during 18 min to led compounds **2** (2.2 mg, Rt= 15.93 min), **5** (4.7 mg, Rt= 14.56 min) and **15** (4 mg Rt= 7.03 min). Semi-prep HPLC on RP-18 of sub-fraction 7 eluted by CH_3CN-H_2O (35:65 - 8:2) during 20 min yield compounds **9** (0.9 mg, Rt= 5.98 min) and **14** (1.8 mg, Rt= 15.99 min)**.** Sub-fraction 8 was purified by semi-prep HPLC using a gradient of solvent CH_3CN-H_2O (3:7- 10:0) during 30 min to provide compound **19** (1.9 mg, Rt= 17.03 min). 2g of the fraction 5 (10:0) was fractioned on silica gel flash chromatography eluted with a gradient of the mixture Hexane-$CHCl_3$-MeOH (10:0:0 - 0:0:10) during 30 min to give 8 sub-fractions. Sub-fraction 2 (38 mg) was submitted to semi-prep HPLC on RP-18 silica gel eluted with CH_3CN/H_2O (8:2) yielding compound **17** (1.9 mg). Sub-fraction 4 (43.5 mg) was purified on RP-18 flash chromatography eluted with a gradient of MeOH-H_2O (6:4 -10:0), the resulting sub-fractions 3 was purified by semi-prep HPLC on RP-18 using a gradient of solvents CH_3CN-H_2O (8:2-10:0) during 15 min to give compound **10** (2 mg, Rt= 11.24 min). Semi-prep HPLC on RP-18 of sub-fraction 7 (23.8 mg) eluting with CH_3CN-H_2O (8:2) during 20 min, allowed compounds **11** (1.5 mg, Rt= 7.30 min), **1** (0.9 mg, Rt= 13.81 min), **4** (0.9 mg, Rt= 14.98 min), and **3** (1.3 mg, Rt= 16.03 min). The sub-fraction 8 (35.7mg) was further purified by semi-prep HPLC on RP-18 by elution with CH_3CN-H_2O (7:3) during 15 min to give compounds **21** (1.2 mg, Rt= 6.53 min) and **13** (5.3 mg, Rt= 10.92 min).

4.3.1. 11*E*-15*E*-19*E*-9-(2,8-dimethyl-5-methoxy-chroman-6-ol)-12,16,20-trimethyl-11,15,19-tridecatrienoic acid (**1**)

White powder; $[\alpha]_D^{20}$ -0.8 (MeOH); ^{1}H NMR (CD_3OD, 600MHz) and ^{13}C NMR (CD_3OD, 150MHz) see Table 1; HRESIMS *m/z* 479.2766 $[M+Na]^+$ (calcd. for $C_{28}H_{40}O_5$, 479.2773).

4.3.2. Isorhamnetin (3,6-*O*-di-*E*-cinnamoyl)-3-*O*-*β*-D-glucopyranoside (**2**)

Yellow powder; ^{1}H NMR (CD_3OD, 600MHz) and ^{13}C NMR (CD_3OD, 150MHz) see Table 1; HRESIMS *m/z* 761.1851 $[M+Na]^+$ (calcd. for $C_{40}H_{34}O_{14}$,761.1846).

4.4 Microbiological Analysis

4.4.1 Culture and preparation of micro-organisms: The 22 bacteria and yeasts used in this study (Table 2) were incubated overnight at 37 °C in tubes containing sloping Mueller-Hinton (MH) agar medium. The bacteria were diluted with MH to 10^5 bacteria/mL by means of serial dilution just before the antimicrobial assays. The same process was performed for the fungi.

4.4.2 MIC determination (solid media): The minimum inhibitory concentration (MIC) was studied using Mueller-Hinton (MH) agar medium in Petri dishes seeded by a multiple inoculator (Abedini et al., 2014). The hydromethanolic extract was tested at six final concentrations (10, 5, 2.5, 1.2, 0.6, and 0.3 mg/mL) against 22 micro-organisms. The agar plates were incubated for 24 hours at 37 °C. The activity was then estimated visually by the presence or absence of colonies. MIC values were recorded as the lowest concentrations of compounds showing no growth. Solvent (methanol 50%) used to prepare the extract was also used solely to checked for absence of antibacterial activity in the test. Three positive controls were used for bacteria (gentamicin and vancomycin) and yeasts (amphotericin B).

4.4.3 Bioautography: To identify the compounds responsible for the antibacterial activity, we adopted an immersion bioautography method (Abedini et al., 2016). An aliquot of each compound (2 mg) was solubilized in 1 mL methanol. The resulting solutions were spotted onto TLC plates (silica gel 60 F254, Merck). Gentamicin (50 µg) was also spotted on the plates as a positive control. The TLC plates were directly dried without migration and sterilized. The plates were then covered by MH agar medium containing a *Staphylococcus aureus* 53.154 suspension (10^5 bacteria/mL) in square Petri dishes. After incubation for 24 h at 37 °C, bacterial growth

was revealed by a 2 mg/mL solution of thiazolylbluetetrazolium bromide (MTT) and growth inhibition zones were measured.

4.4.4 MIC determinations by broth microdilution method: A serial dilution technique using 96-well microtiter plates was used to determine the MIC of the most active compounds against five sensitive bacteria (Abedini et al., 2016). Nine concentrations of each compound, from 500 µg/mL to 1.9 µg/mL, were used. They were serially twofold diluted in nine wells. Three wells were represented as bacteria culture control (positive control), medium sterility control (negative control) and medium with solvent (solvent basal activity) to check for absence of antibacterial activity. Then the wells were loaded with MH liquid medium and bacterial suspension (10^5 bacteria/mL) giving a final volume of 200 μL. The plates were incubated overnight at 37°C. Bacterial growth was followed visually and then by direct spray of 0.2 mg/mL MTT to each well and the plates was incubated at 37°C for at least 10 minutes. MIC values were determined as the lowest concentrations of compounds showing clear wells. This test was performed in triplicate.

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Appendix A. Supplementary data

Figure of isolated compounds (**1-21**) and spectral data of the new compounds **1** and **2** associated with this article can be found, in the online version, at http://

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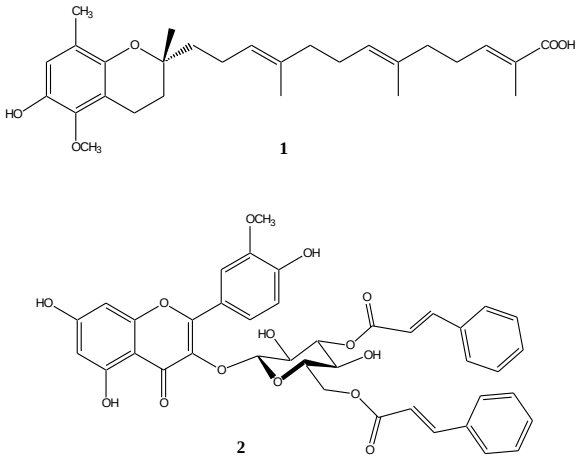

Fig. 1. Structures of compounds **1** and **2**

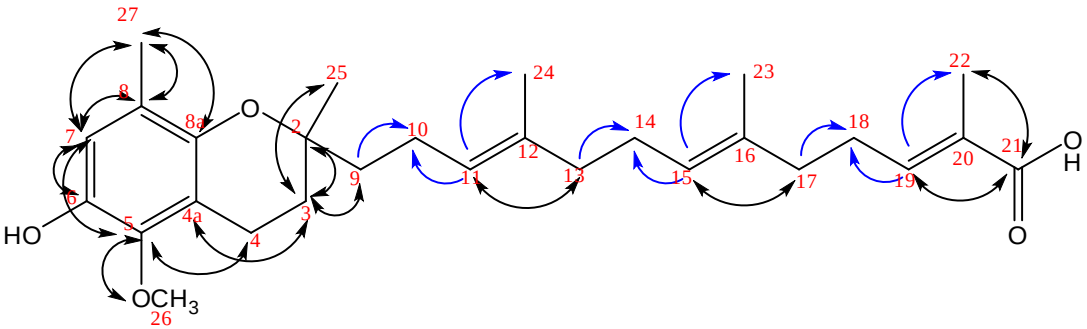

Fig. 2. COSY (→) and HMBC (↔) correlations for compound **1**

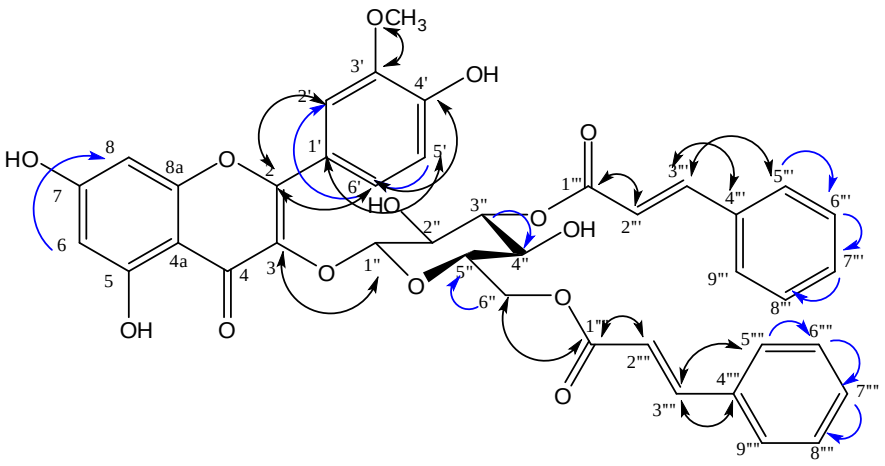

Fig. 3. COSY (→) and HMBC (↔) correlations for compound **2**

Table 1: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of compounds **1** and **2** in MeOD

Atom	**1**		Atom	**2**	
	δ_H, mult.,*J* (Hz)	δ_C		δ_H, mult.,*J* (Hz)	δ_c
1			1		
2	-	75.9	2	-	157.5
3	1.71, dd, 13.7, 6.8 1.79, dd, 13.7, 6.8	32.1	3	-	133.7
4	2.73, td, 6.8, 1.5	18.4	4	-	177.9
4a	-	116.1	4a	-	104.2
5	-	144.4	5	-	161.6
6	-	142.8	6	6.15, d, 1.9	98.6
7	6.51, s	117.1	7	-	164.6
8	-	122.4	8	6.31, d, 1.9	93.4
8a	-	146.2	8a	-	157.0
9	1.56, m 1.62, m	40.3	9	-	-
10	2.14, q, 7.8	23.2		-	-
11	5.27, t, 7.6	125.9	1'	-	121.5
12	-	135.8	2'	7.85, d, 1.9	112.8
13	2.01, t, 7.3	40.7	3'	-	147.0
14	2.10, m	27.5	4'	-	149.7
15	5.25, t, 7.4	126.2	5'	6.89, d, 8.4	122.6
16	-	135.1	6'	7.60,dd, 8.4, 1.9	114.6
17	2.10, m	39.4	7'	3.95,s	55.3
18	2.29, q, 7.4	28.3	1"	5.48, d, 7.9	102.2
19	6.74, t, 6.6	143.0	2"	3.75, dd, 9.3, 7.9	72.8
20	-	129.5	3"	5.20, t, 9.3	74.3
21	-	172.4	4"	3.62, t, 9.7	68.9
22	1.82, s	12.6	5"	3.68, m	77.6
23	1.62, s	16.0	6"	4.35, m	62.7
24	1.60, s	15.9	1"'	-	166.9
25	1.27, s	24.4	2"'	6.66, d, 16.0	117.7
26	3.76, s	60.4	3"'	7.80, d, 16.0	145.1
27	2.06, s	15.9	4"'	-	134.5
			5"'	7.66, m	127.9
			6"'	7.44, m	128.6
			7"'	7.43, m	130.1
			8"'	7.44, m	128.6
			9"'	7.66, m	127.9
			1""	-	166.6
			2""	6.29, d, 16.2	116.8
			3""	7.48, d, 16.2	145.0
			4""	-	134.2
			5""	7.48, m	127.9
			6""	7.42, m	128.6
			7""	7.43, m	130.1
			8""	7.42, m	128.6
			9""	7.48, m	127.9

Table 2**.** Minimum inhibitory concentration (MIC) of hydromethanolic extract of *Cedrus atlantica* cones (by solid medium).

Micro-organisms	MIC (mg/mL)	Positive controls		
		G	**V**	**A**
Gram positive bacteria				
Bacillus subtilis	≤ 0.3	S	S	NT
*Enterococcus faecalis*ATCC 1034	≤ 0.3	R	R	NT
Staphylococcus aureus 8325-4	1.2	S	S	NT
Staphylococcus aureus CIP 53.154	1.2	S	S	NT
Micrococcus luteus	≤ 0.3	S	S	NT
Listeria innocua	1.2	S	S	NT
Streptococcus pyogenes	≤ 0.3	S	S	NT
Staphylococcus epidermidis	0.6	S	S	NT
Gram negativebacteria				
Escherichia coli CIP 54.127	NA	S	R	NT
Enterobacter cloacae	NA	S	R	NT
Salmonella enterica	NA	S	R	NT
Serratia marcescens	NA	S	R	NT
Proteus vulgaris	10	S	R	NT
Klebsiella pneumoniae	NA	R	R	NT
Providencia stuartii	10	S	R	NT
Pseudomonas aeruginosa ATCC 9027	10	S	R	NT
Shigella soneii	≤ 0.3	S	I	NT
Yeast				
Candida glabrata	1.2	R	R	S
Candida tropicalis	NA	R	R	S
Candida kefyr	NA	R	R	S
Candida albicans	NA	R	R	S
Cryptococcus neoformans	NA	R	R	S

NA: no activity, NT: not tested;

S: sensitive; I: intermediate sensitivity; R: resistant. MIC (µg/mL) of positive controls: **G**entamicin, S: ≤4, R: >8; **V**ancomycin, S: ≤4, R: >16; **A**mphotericin B, S: ≤1, R: >4.

Table 3**.** Antibacterial activity of the purified compounds by bioautography and liquid medium.

		MIC (µg/mL)				
Compound / ATB	Bioautography against *S. aureus* CIP 53.154	*E. faecalis* ATCC 1034	*S. aureus* CIP 53.154	*S. epidermidis*	*E. coli* CIP 54.127	*P. aeruginosa* ATCC 9027
1	++	125	125	250	500	500
3	+++	62.5	125	125	250	125
5	++	250	250	62.5	250	125
6	+++	125	250	250	250	250
7	++	125	125	62.5	250	250
9	+	250	250	500	NA	NA
10	++++	31.2	31.2	125	250	250
11	++++	31.2	62.5	125	250	125
13	+++	62.5	62.5	125	250	250
14	++++	31.2	31.2	250	250	250
15	+++	62.5	125	31.2	125	62.5
16	++++	31.2	62.5	125	125	250
17	++++	15.1	31.2	125	125	125
18	+++	62.5	125	250	250	250
20	++++	62.5	62.5	125	125	250
21	++	500	250	NA	NA	NA
Gentamicin	++	15.1^{R}	0.5	0.25	4	8
Vancomycin	NT	125^{R}	4	4	125^{R}	125^{R}

(+): significant effect, rated from + (0.5 cm inhibition zone) to ++++ (> 1.5 cm inhibition zone); NA: no activity; NT: not tested; ^{R}: resistant.