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Chemical composition, antioxidant and antibacterial activities of aerial parts of *Tamarix balansae* J. Gay aerial parts

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Abstract

A previously undescribed phenolic sulfate ester, potassium 3,4-dihydroxy-3-methoxybenzoic acid methyl ester-5-sulfate (**1**), along with nine known flavonoids, kaempferol-3-*O*-potassium sulfate-4',7-dimethyl ether (**2**), kaempferol-4',7-dimethyl ether (**3**), rhamnocitrin-3-*O*-potassium sulfate (**4**), rhamnocitrin (**5**), kaempferol (**6**), quercetin (**7**), afzelin (**8**), quercetin-3-*O*-*α*-L-rhamnopyranoside (**9**) and luteolin-3'-*O*-potassium sulfate (**10**) were isolated from the aerial parts of *Tamarix balansae*. Structures elucidation was performed by comprehensive 1D and 2D NMR analyses, mass spectrometry and by comparison with literature data. The antibacterial assay against *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis, Escherichia coli* and *Pseudomonas aeruginosa* bacteria showed a good activity for **2**, **3**, **7** and **9**, with MICs ranging from 62.5 to 250 µg/mL. The abilities of these compounds to scavenge the DPPH were evaluated. Compounds **1**, 7, **9** and **10** exhibited a good antiradical activity potential with IC_{50} values ranging from 3.0 to 115.5 µg/mL, compared with ascorbic acid (IC_{50} 7.4 µg/mL) which was used as positive control.

Keywords: *Tamarix balansae*, Tamaricaceae, flavonoids, antibacterial activity, antioxidant activity

Abbreviations:

MH: Mueller-Hinton; MIC: Minimum Inhibitory Concentration; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) bromide; DPPH: 2,2-diphenyl-1-picrylhydrazyl.; DMSO: Dimethysulfoxide

1. Introduction

Tamaricaceae is a relatively small family containing four genera and about 125 species (Trease and Evans, 2002). *Tamarix*, represented by approximately 50 species, is the largest genus in this family. *Tamarix* plants are distributed from Morocco to India (Sultanova et al. 2001) with 10 species growing in Algeria (Quezel et Santa, 1963). *Tamarix balansae* J. Gay is an endemic Saharan plant growing in salty areas. Its leaves are long and wide, rarely slightly longer acute mucronate at the top, and the flower is colour pinkish-white to purple, small (4-5 mm), with capsules opening with 3 valves (4-5) mm, the etamines (10) are inserted on the horns of the disc (Quezel et Santa, 1963). *Tamarix* species have been used in traditional medicine, especially as antiseptic agents and to treat leucoderma, spleen trouble and eye diseases (Nawwar et al., 1982). Polyphenolic compounds including, flavonoids (Lefahal et al. 2010, Mahfoudhia et al. 2014), phenolic acids (Akhlaq et al. 2012), tannins and lignans (Souliman et al 1991), represent the most common phytochemically investigated compounds in *Tamarix* species. Various species have been reported to possess antioxidant (Sultanova et al. 2001, Mohammedi et al. 2012), antimicrobial (Sultanova et al. 2001, Lefahal et al. 2010) or cytotoxic (Bakr et al. 2013) activities. Very recently, the aqueous extract of *T. aphylla* leaves has been used in an eco-friendly dyeing process of cotton modification (Baaka et al. 2017). The present study describes the isolation and the structure elucidation of a previously undescribed phenolic sulfate (**1**) and nine known flavonoids (**2-10**) from the aerial parts of *Tamarix balansae*. Their structural assignments were made by ESI-MS, 1D and 2D NMR analyses. As mentioned above, several *Tamarix* species have been reported to possess antioxidant and antibacterial activities, therefore DPPH radical scavenging inhibitory activity and antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli* of the constituents from the aerial parts of *T. balansae* were evaluated.

2. Results and discussion

The *n*-BuOH soluble fraction of the MeOH 80% extract of dried aerial parts of *T. balansae* was fractionated and purified by combination of chromatographic methods to obtain a new phenolic sulfate (**1**) and nine known compounds (**2**-**10**). The known compounds were elucidated as kaempferol-3-*O*-potassium sulfate-4',7-dimethyl ether (**2**) (Nawwar et al. 1984), kaempferol-4',7-dimethyl ether (**3**) (Rossi et al. 1997), rhamnocitrin-3-*O*-potassium sulfate (**4**) (Fang et al. 2008), rhamnocitrin (**5**) (Mann et al. 1999), kaempferol (**6**) (Gangwal et al. 2010), quercetin (**7**) (Touafek et al. 2011), afzelin (**8**) (Granja-Pérez et al. 1999), quercetin-3-*O*-*α*-L-rhamnopyranoside (**9**) (Zhang et al. 2014) and luteolin-3'-*O*-potassium sulfate (**10**) (Baron and Ibrahim, 1988) (Fig. 1).Their spectroscopic data were in perfect agreement with those reported in the literature.

Compound **1** was obtained as a white amorphous powder. The positive ESI-MS spectrum (Fig. S1) showed an $[M+Na]^+$ ion at *m/z* 339, compatible with the molecular formula of $C_9H_9O_8SK$. The UV spectrum (Fig. S2) revealed an absorption band at 276 nm (MeOH). In the IR spectrum (Fig. S3), in addition to the obvious evidence for a hydroxyl (3324 cm^{-1}), carbonyl (1708 cm^{-1}) and aromatic ring (1606, 1517 cm^{-1}), the strong absorption at 1232 and 1054 cm^{-1} was indicative of the presence of an $–O-SO_3^-$ group (Barron et al., 1988). The presence of the sulfate group was confirmed by precipitation with $BaCl_2$, after acid hydrolysis of **1** (Xiao et al., 2002).

The ^1H and ^{13}C-NMR spectra of compound **1** suggested a tetrasubstituted phenolic compound. Its aromatic ring was represented by signals for a pair of *meta*-coupled protons resonating at δ_H 7.42 and 7.76 (each 1H, d, J =2.0 Hz). In addition, characteristic signals for a carbonyl group (δ_C 168.2), a methoxy group [δ_H 3.89 (s, 3H) and δ_C 56.7)], a methyl ester [δ_H 3.86 (s, 3H) and δ_C 52.3)] and three signals of oxygenated aromatic carbons at δ_C 141.5, 145.4 and 150.0 were observed (Table S1). Further interpretation of 2D NMR especially HMBC data established that compound **1** was a gallic acid derivative substituted by methoxy, methyl ester and sulfate groups (Xiao et al. 2002) (See supporting material, Fig. S8). The HMBC spectrum showed correlations between the methoxy group (δ_H 3.89) and C-3 (δ_C 150.0), which allowed the assignment of OCH_3 in position C-3 whereas correlations between the methyl ester group (δ_H 3.86) and the carbonyl carbon placed the methyl ester group in position C-7 (δ_C 168.2). Compound **1** was similar to gallic acid-3-methyl ether-5-sodium sulfate, previously isolated from *Frankenia laevis* L. (Hussein 2004). The only difference was in the presence of a methyl ester group in **1** and the nature of the sulfate moiety. Thus, compound **1** was identified to be potassium 3,4-dihydroxy-3-methoxybenzoic acid methyl ester-5-sulfate.

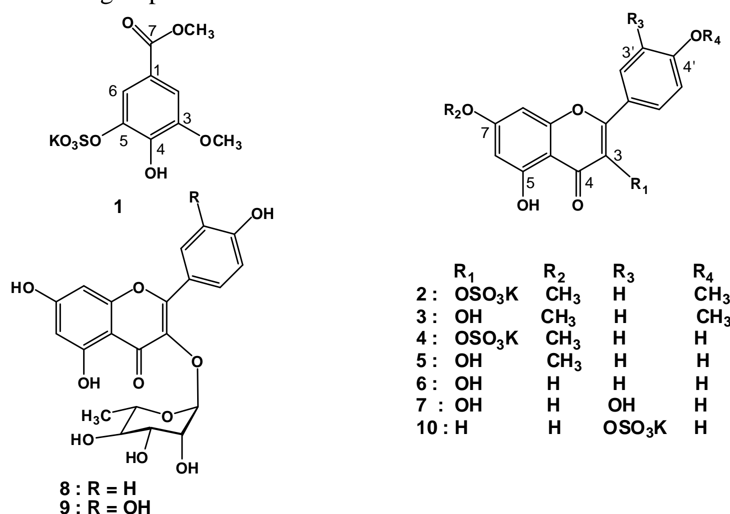

Figure 1: Chemical structures of compounds **1**-**10** isolated from *T. balansae.*

Table S1. NMR spectral data of compound 1 and gallic acid-3-methyl ether-5-sodium sulfate.

	Compound 1				Gallic acid-3-methyl ether-5-sodium sulfate	
	δ_H m (J Hz)	δ_C	^1H-^1H COSY	HMBC (H to C)	δ_Hm (J Hz)	δ_C
1	-	121.2	-		-	119.6
2	7.42 d (2.0)	110.7	H-6	C-1, 3, 5, 6, 7	7.20 d (2.5)	108.9
3	-	150.0		-	-	149.1
4	-	141.5	-	-	-	141.6
5	-	145.4	-	-	-	144.1
6	7.76 d (2.0)	118.7	H-2	C-2, 4, 5, 7	7.60 d (2.5)	117.4
7	-	168.2	-	-	-	166.7
3-OCH₃	3.89 s	56.7	-	C-3	3.80	-
7-OCH₃	3.86 s	52.3	-	C-7	-	-

The *n*-BuOH soluble fraction of *T. balansae* was initially tested for its antibacterial capacity against *Staphylococus aureus* CIP 53.154 by using TLC bioautography method (Benmerache et al., 2016).The results of agar-overlay bioautography revealed five inhibition zones on the developed extract. Each zone contains one or several antibacterial compounds. Subsequently, the *n*-BuOH soluble fraction was then fractionated and purified by combination of chromatographic methods to obtain ten compounds (**1-10**) and a second bioautography with compounds **1-10** was performed (Table S2). Compounds **2**, **3**, **7** and **9** showed a good antibacterial activity against *S. aureus* with 0.5 to 1.5 cm inhibition zone diameter.

A serial dilution technique using 96-well microtiter plates was used to determine the minimum inhibitory concentration values (MIC) of compounds **3, 7** and **9** 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 (Table S2). These compounds showed a good antibacterial activity against the five bacteria. Compound **2** was the most active with MIC values of 62.5 µg/mL against *E. faecalis*, *S. aureus*, *S. epidermidis* and *P. aeruginosa* and 125 µg/mL against *E. coli*. Compound **9** also inhibited *E. faecalis*, *S. epidermidis* and *P. aeruginosa* (MIC 62.5 µg/mL), *S. aureus* (MIC 125 µg/mL) and *E. coli* (MIC 250 µg/mL). Compound **3** showed a moderate antibacterial activity against the five tested bacteria with MIC values ranging from 125 to 250 µg/mL. The antibacterial potential of compound **7** was good against *E. faecalis* and *P. aeruginosa* (MIC 62.5 µg/mL), moderate against *S. epidermidis* and *S.aureus* (MIC 125 µg/mL) and low against *E. coli* (MIC 500 µg/mL). Comparison of the antibacterial activity of kaempferol-4',7-dimethyl ether derivatives (**2** and **3**) suggested that potassium sulfate was favorable for the antibacterial activity in **2**. In the same manner, comparison of the antibacterial activity of quercetin derivatives (**7** and **9**) suggested that the substitution by a rhamnopyranose residue (**9**) increases the antibacterial activity.

Table S2. Antibacterial activity of compounds 1-10 isolated from *Tamarix balansae*.[a]

		MIC (µg/mL)				
	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
2	++	62.5	62.5	62.5	125	62.5
3	++	125	250	250	250	250
7	+++	62.5	125	125	500	62.5
9	+	62.5	125	62.5	250	62.5
Gentamicin[b]	++	R	S	S	S	S

[a]No activity was detected for compounds **1**, **5**, **6**, **8** and **10** by bioautography

[b] used as positive control

(+): significant effect, rated from + (0.5 cm inhibition zone) to ++++ (> 1.5 cm inhibition zone); R: resistant; S: sensitive. MIC (µg/mL) of positive control Gentamicin, S: ≤4, R: >8.

The *n*-BuOH soluble fraction exhibited a mild DPPH radical scavenging activity (IC_{50} 48 µg/mL) compared to ascorbic acid, used as a positive control. The DPPH radical scavenging activity of compounds isolated from this fraction (**1-10**) was measured (Table S3). Compounds **7** and **9** possess an excellent antiradical activity (IC_{50} 3.01 and 13.6 µg/mL, respectively) comparable to ascorbic acid, used as a positive control (IC_{50} 7.4 µg/mL). Compounds **1** and **10** showed a low to moderate DPPH radical scavenging activity (IC_{50} 115.5 and 55.3µg/mL, respectively). The seven other compounds did not show scavenging activity at 200 µg/mL. Generally, substitution patterns of flavonoids on the B-ring especially affect antioxidant potencies of the flavonoids (Arora et al., 1998). Flavonols and flavones containing a catechol group in ring B are highly active, and flavonols are more potent than the corresponding flavones because of the presence of the 3-hydroxyl group (Pietta et al., 2000). These trends are consistent with the active compounds **7** and **9** possessing 3,3′,4′-OH pattern. Glycosylation of 3-hydroxyl group in compound **9** reduces the radical-scavenging capacity compared to compound **7**. The lack of 3-hydroxyl group in compound **10** reduced the activity.

Table S3. DPPH radical scavenging activities of the *n*-BuOH soluble fraction and compounds 1-10 isolated from *Tamarix balansae*.[a]

	IC_{50} ± S.D. (µg/mL)
***n*-BuOH fraction**	48.0
1	115.5
7	3.01
9	13.6
10	55.3
Ascorbic acid[b]	7.4 ± 0.2

[a]50% inhibition for compounds **2-6** and **8** not achieved at the concentration of 200 µg/mL

[b] used as positive control.

3. Experimental

3.1. General experimental procedures

The optical rotations were recorded on a Perkin-Elmer 341 Polarimeter. The UV spectra were obtained in MeOH on a Shimadzu UV-2450 spectrophotometer. IR spectra were obtained using a Shimadzu IR-470 spectrometer. 1D and 2D NMR spectra were recorded in CD_3OD on a Bruker Avance DRX III 500 spectrometer (^1H at 500 MHz and ^{13}C at 125 MHz). ESI-MS experiments were performed using a Micromass Q-TOF instrument. Silica gel 60 F_{254}pre-coated aluminium plates (0.2 mm, Merck) were used for TLC analysis. The TLC and prepTLC spots were visualized under UV light (254 and 366 nm) followed by spraying with 50% H_2SO_4 and heating. Column chromatography was carried out on Kieselgel 60 (63-200 mesh) Merck. Biological assays were read on a Fluostar Omega microplate reader (BMG labtech).

3.2. Plant material

Tamarix balansae J. Gay was collected from In Amenas (Illizi, South Eastern Algerian) in April 2013, during the flowering stage and authenticated by Mr. K. Kabouche. A voucher specimen has been deposited in the Herbarium of the laboratory of Therapeutic Substances (LOST), University frères Mentouri-Constantine (LOST Tb/04/13).

3.3. Extraction and isolation

The powdered dried aerial parts (1.5 kg) of *Tamarix balansae* were macerated at room temperature in MeOH 80% (3×7L, 24h). The extract was concentrated under low pressure, the residue (120 g) was dissolved with water (1L), and filtered, then successively extracted with dichloromethane and *n*-BuOH (3×300 mL each). After evaporation of the solvents, 1.5 g of CH_2Cl_2 and 16 g of *n*-BuOH extracts were obtained.

A part of *n*-BuOH extract (12 g) was subjected to column chromatography on polyamid SC6 (90×5.5 cm), with a stepwise gradient of toluene-MeOH (10:0, 0:10) as eluent and 23 fractions of 250 mL were collected. Fraction 3 was purified by column chromatography on silica gel, eluted with $CHCl_3$ to afford compound **2** (10 mg). Fraction 6 was subjected to a silica gel column chromatography eluted with EtOAc:MeOH (9:1) leading to seven subfractions, the subfraction f3 was obtained as a yellow precipitate compound **3** (4 mg). Fraction 7 was purified by silica gel preparative TLC (20×20 cm) with EtOAc-MeOH (95:5) as eluent to give compound **4** (5 mg). Fraction 9 was column chromatographed on silica gel, eluting with an isocratic system of EtOAc:MeOH (8:2) leading to nine subfractions, the subfraction f4 was separated by silica gel column chromatography eluted with an isocratic system of EtOAc:MeOH:H_2O (20:2:1) affording compounds **5** (4 mg) and **6** (8 mg). Fraction 14 was subjected to a silica gel column chromatography eluted with EtOAc:MeOH with increasing polarity leading to nine subfractions, the two subfractions f7 and f9 were purified by silica gel column eluted with an isocratic system of EtOAc:MeOH:H_2O (20:2:1) affording compounds **7** (7 mg) and **8** (6 mg). Fraction 17 was subjected to a silica gel column chromatography eluted with EtOAc:MeOH with increasing polarity to give compounds **9** (4 mg) and **10** (5 mg). Fraction 21 containing one major spot was column chromatographed on silica gel eluting with an isocratic system of EtOAc:MeOH:H_2O (20:2:1) leading to five subfractions, the subfraction f7 was obtained as a white precipitate compound **1** (18 mg).

3.3.1. Potassium 3,4-dihydroxy-3-methoxybenzoic acid methyl ester-5-sulfate (1)

Amorphous powder, $[\alpha]_D^{20}$ +187.5 (*c* 0.3 MeOH). ESI-MS : *m/z* 339.0 $[M+Na]^+$. UV λ_{max} (MeOH, nm): 276 (sh), IR (KBr) cm^{-1}: 3324, 1708, 1606, 1517, 1439, 1355, 1278, 1232, 1201, 1086, 1054, 1005.^1H NMR (500 MHz, CD_3OD) : δ_H 7.76 (d, J = 2.0 Hz, H-6), 7.42 (d, J = 2.0 Hz, H-2), 3.89 (s, 3-OCH_3), 3.86 (s, 7-OCH_3).^{13}C NMR (125 MHz, CD_3OD) : δ_C 168.2 (C-7), 150.0 (C-3), 145.4 (C-5), 141.5 (C-4), 121.2 (C-1), 118.7 (C-6), 110.7 (C-2), 56.7 (3-OCH_3), 52.3 (7-OCH_3).

3.3.2. Acid Hydrolysis.

Solutions of **1**, **2**, **4** and **10** (2 mg each) in 2M HCl were heated at 100 °C for 1 h. After cooling to room temperature, the hydrolysate was extracted with EtOAc. The recrystallized material was identified by its UV spectra in MeOH and by comparison of its *Rf* with authentic samples. $BaCl_2$ was added to a sample of each aqueous layer, resulting in the formation of a white precipitate for compounds **1**, **2**, **4** and **10**, confirming the presence of a sulfate moiety (Xiao et al., 2002).

3.4. Antibacterial Activity

Two methods were used to find and determinate this activity: bioautography and broth microdilution

3.4.1. Evaluation of the antibacterial activity of compounds against S. aureus by bioautography

At first to find out the antibacterial compounds, an immersion bioautography method was adopted (Abedini et al. 2013). An aliquot of each compound (2 mg) was solubilized in 1 mL of MeOH. The resulting solutions (25 µL) were spotted onto Merck 60 F_{254} pre-coated silica gel plates (10x10 cm). 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 Mueller-Hinton (MH) agar medium containing a *S. aureus* suspension (10^5 bacteria/mL) in square Petri dishes. After incubation 24 h at 37 °C, bacterial growth was revealed by a 2 mg/mL solution of MTT and growth inhibition zones were measured. White stains indicate where reduction of MTT to the colored formazan did not take place due to the presence of compounds that inhibited bacterial growth.

3.4.2. MIC determination of the most active compound against five micro-organisms by broth microdilution

A serial dilution technique using 96-well microliter plates was used to determine the MIC values of the most promising compounds as revealed by bioautography (Abedini et al. 2016). For this purpose, nine concentrations of the active compounds, from 500 µg/mL to 1.9 µg/mL, were tested. Two wells were represented as bacteria culture control (positive control) and medium sterility control (negative control). 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, sprayed with a 0.2 mg/mL MTT solution and incubated again at 37 °C for 30 min. Bacterial growth was indicated by a violet color whatever the color intensity, while bacterial growth inhibition was admitted only for wells which remained clear. MIC values were determined as the lowest concentrations of samples having an inhibitory effect on bacteria growth (clear wells). This test was performed in triplicate.

3.5. DPPH radical scavenging activity

The scavenging activity of isolated compounds against DPPH was investigated by spectrophotometric methodology, as previously described (Benmerache et al., 2016). Briefly, 5 µL of either standard or sample solutions (dissolved in DMSO) was mixed with 95 µL of DPPH solution (158 µM, dissolved in absolute EtOH). After mixing gently and incubating for 30 min at 37 °C, the optical density was measured at λ 515 nm. The % scavenging activity was calculated using the following equation: % inhibition $[(Ab_{control} - Ab_{sample})/Ab_{control}] \times 100$. DPPH solution in EtOH was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by the MS Excel based program to obtain the IC_{50}. All the tests were conducted in triplicate. The experimental data were expressed as mean ± standard deviation.

4. Conclusions

One new phenolic sulfate ester, along with nine known flavonoids, were isolated from the aerial parts of *T. balansae*. Compounds **2**, **3**, **7** and **9** showed antibacterial activity against *E. faecalis*, *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli* with MICs ranging from 62.5 to 500 µg/mL. The DPPH radical scavenging activity assay showed a high activity for compound **7** compared with ascorbic acid used as a positive control. Compounds **1**, **9** and **10** exhibited a mild to low DPPH radical scavenging activity.

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Supporting Information

Antibacterial and DPPH radical scavenging activities of compounds **1-10** ESI-MS, IR, UV, and NMR data for compound **1**

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