New sulphated flavonoids from *Tamarix africana* and biological activities of its polar extract

Manel Karker[1], Nunziatina De Tommasi[2], Abderrazak Smaoui[3], Chedly Abdelly[3], Riadh Ksouri[1], Alessandra Braca[4,5*]

Affiliation

[1] Laboratory of Aromatic and Medicinal Plants, Biotechnology Center at the Technopole of Borj-Cédria (CBBC), Hammam-Lif, Tunisia

[2] Dipartimento di Farmacia, Università di Salerno, Fisciano (SA), Italy

[3] Laboratory of Extremophile Plants, Biotechnology Center at the Technopole of Borj-Cedria (CBBC), Hammam-Lif, Tunisia

[4] Dipartimento di Farmacia, Università di Pisa, Pisa, Italy

[5] Centro Interdipartimentale di Ricerca "Nutraceutica e Alimentazione per la Salute", Università di Pisa, Pisa, Italy

Correspondence:

Prof. Alessandra Braca, Dipartimento di Farmacia, Università di Pisa, Via Bonanno 33, 56126 Pisa, Italy. E-mail: alessandra.braca@unipi.it. Phone: +39 050 2219688 Fax: +39 050 2220680

Abstract

The phytochemical investigation of *Tamarix africana* Poir. (Tamaricaceae) shoot polar extract afforded three new sulphated flavonoids, (2*S*,4*R*)-5,7,4'-trihydroxyflavan-4-ol 5,7-disulphate (**1**), (2*S*)-5,7,4'-trihydroxyflavan 7-*O*-sulphate (**2**), and (2*S*)-naringenin 4'-*O*-sulphate (**3**), together with ten known compounds. Their structures were determined by spectroscopic methods including 1D and 2D NMR analysis and high resolution mass spectrometry. Biological activities of the polar extract of *T. africana* shoots related to its phenolic content were also investigated. A high total phenolic content (151.1 mg GAE/g) was found in the methanol shoot extract, which exhibits strong antioxidant activities using the ORAC method and in a skin cell based-assay. Moreover, the shoot extract showed significant anti-inflammatory activity, reducing NO release by 53.5 % at 160 µg/mL in LPS stimulated RAW 264.7 macrophages. Finally, *T. africana* shoot extract inhibited the growth of A-549 lung carcinoma cells, with an IC_{50} value of 34 µg/mL.

Key words

Tamarix africana, Tamaricaceae, sulphated flavonoids, antioxidant activity, cytotoxicity, anti-inflammatory activity

Introduction

The genus *Tamarix* (commonly known as tamarisk, Tamaricaceae family) comprises green tree halophytes, growing to 1-18 m tall, mainly found in coastal saline soil and desert [1]. They are long-living plants very tolerant to severe conditions and sustain abiotic stresses, such as high temperature, salt and dryness [2]. Some species are cultivated as ornamental plants in gardens or as windbreaks or shade trees [3]. *Tamarix* species synthesize and accumulate a large variety of phenolics, including lignans, phenolic glycerides, gallotannins, monomeric and oligomeric ellagitannins, flavonol glycosides, coumarins, and fatty alcohols [4-6]. In addition, they are used in traditional medicine as astringent, aperitif, stimulus of perspiration and diuretic [7], and are used for tanning and dyeing purposes [8, 9]. Younos et al. [10] reported the importance of this genus in some old civilizations and the actual uses of different parts of *Tamarix* species (leaves, flowers, and galls) in the traditional Asian medicine as anti-inflammatory, anti-diarrheal, cicatrizing, and antiseptic agents.

It is well known that plant halophytes produce polyphenols as stress metabolites and could be a source of bioactive compounds [2, 11]. Therefore, *T. africana* Poir, an ornamental and folkloric medicinal halophyte, particularly abundant in Mediterranean salt marshes [7], was selected for this study. *T. africana* is a perennial shrub with brownish branches, punctuated, opaque, oval and acuminate leaves; its bunches reach 6 cm long and contain flowers with four to five petals and four stamens. To our knowledge, no detailed study on the chemical composition or any biological activities of *T. africana* shoots have been reported in the literature. The present investigation was designed to carry out a phytochemical study of *T. africana* shoots and to evaluate the biological properties of the methanol extract.

Results and Discussion

The methanolic shoot extract of *T. africana* was subjected to Sephadex LH-20 column chromatography followed by flash chromatography on SiO_2 and RP-HPLC, to afford three new sulphated flavonoids (**1-3**) (**Fig. 1**), along with ten known compounds.

Compound **1** was obtained as red-brownish amorphous powder. Its UV spectrum showed two absorption maxima at 254 and 296 nm. Its molecular formula was assigned as $C_{15}H_{13}NaO_{11}S_2$ on the basis of its ^{13}C NMR and HRESIMS (*m/z* 454.9733 $[(M-H]^-)$ data. The HRESIMS/MS spectrum showed two peaks at *m/z* 335.02 $[(M-120)-H]^-$ and 255.07 $[(M-120-80)-H]^-$ suggesting the presence of a sulphate group. Another sodium sulphate residue was ascertained from the fragment at *m/z* 152.01 $[(M-120-80-103)-H]^-$. The ^1H NMR spectrum (**Table 1**), showing four aromatic signals at δ 6.82 (two overlapped signals), 7.03 (1H, d, J = 2.0 Hz), and 7.30 (2H, d, J = 8.6 Hz), two methines at δ 5.20 (1H, dd, J = 12.9, 2.0 Hz) and 5.17 (1H, br s), and one methylene at δ 2.13 (1H, br dd, J = 16.0, 2.0 Hz) and 1.98 (1H, br dd, J = 16.0, 12.0 Hz) was indicative of a flavan-4-ol-type skeleton [12]. The chemical shifts of the two doublets at δ 7.30 (2H, d, H-2'/6') and 6.82 (2H, d, H-3'/5') suggested a *para*-substituted B-ring, while the two doublets at δ 7.03 (1H, d, J = 2.0 Hz, H-6) and 6.82 (1H, d, J = 2.0 Hz, H-8), were in accordance with a 1,2,3,5-tetrasubstituted A-ring. Complete assignment of proton and carbon chemical shifts of **1** was accomplished by HSQC, HMBC, and DQF-COSY. Keys HMBC correlations were observed between δ 5.20 (H-2) and 132.9 (C-1') and 128.6 (C-2'/6'), δ 2.13 (H-3a) and 1.98 (H-3b) and 60.0 (C-4) and 116.2 (C-10), δ 6.82 (H-8) and 7.03 (H-6) and 153.6 (C-7). In order to establish the configuration at C-2 and C-4, the CD spectrum and chemical shifts and coupling constants of H-2, H-3, and H-4 were studied. The coupling constants of H-2 and H-4 (**Table 1**) suggested a *trans* stereochemical relationship between H-2 and H-4. Moreover, the positive Cotton effects at 230 and 275 nm enabled to establish the 2*S*,4*R* configuration [13]. Therefore, compound **1** was characterized as (2*S*,4*R*)-5,7,4'-trihydroxyflavan-4-ol 5,7-disulphate (=(2*S*,4*R*)-5,7,4,4'-tetrahydroxyflavan 5,7-disulphate).

Compound **2** was isolated as a red-brownish amorphous powder. It showed a $[M-H]^-$ ion at *m/z* 337.0235 in the HRESIMS spectrum corresponding to the molecular formula $C_{15}H_{14}O_7S$. In the HRESIMS/MS spectrum fragments at *m/z* 257.14 $[(M-80)-H]^-$, 217.06 $[(M-120)-H]^-$, and 137.15 $[(M-80-120)-H]^-$ were observed, suggesting the presence of a sulphate moiety. The UV spectrum showed a UV maximum at 287 nm indicating a flavan skeleton [14]. The ^1H NMR spectrum (**Table 1**) showed signals for one methine and two methylenes for the C ring at δ 4.88 (overlapped signal), 2.93 (1H, br dd, *J* = 13.0, 1.0 Hz, H-4a) and 2.80 (1H, m, H-4b), 2.13 (1H, m, H-3a) and 1.95 (1H, m, H-3b), two *meta*-coupled aromatic protons at δ 6.65 (1H, d, *J* = 1.8 Hz, H-6) and 6.14 (1H, d, *J* = 1.8 Hz, H-8), and a pair of signals for a 1,4-disubstituted aromatic ring at δ 7.24 (2H, d, *J* = 8.5 Hz, H-2'/6') and 6.80 (2H, d, *J* = 8.5 Hz, H-3'/5'). These signals correlated in the HSQC spectrum with the respective carbons at δ 78.9 (C-2), 21.0 (C-4), 30.0 (C-3), 102.3 (C-6), 101.0 (C-8), 128.5 (C-2'/6'), and 116.1 (C-3'/5'). The sulphate group was located at C-7 on the basis of the chemical shifts of H-6/C-6 and H-8/C-8 and HMBC correlations between H-6 and C-7, C-8, and C-10. The absolute configuration at C-2 was determined on the basis of CD spectroscopic analysis. Compound **2** showed a negative Cotton effect at 280 nm indicating the *S* configuration at C-2 [13]. The structure of **2** was therefore identified as (2*S*)-5,7,4'-trihydroxyflavan 7-*O*-sulphate.

Compound **3** was isolated as a yellow amorphous powder and its molecular formula established as $C_{15}H_{12}O_8S$ based on the ESIMS and ^{13}C NMR experiments. Its UV (two maxima at 287 and 327 nm) and NMR spectra (**Table 1**) resembled those of naringenin [15] with the following difference: the chemical shifts of proton and carbon signals of ring B (δ 7.48 in **3** *versus* 7.32 in naringenin; 7.36 in **3** *versus* 6.81 in naringenin; δ 128.3 in **3** *versus* 129.0 in naringenin, 122.6 in **3** *versus* 116.3 in naringenin) were indicative of the presence of a sulphate group at C-4' in **3**. The configuration at C-2 was determined as *S* on the basis of a negative Cotton effect at 282 nm and a positive Cotton effect at 330 nm in the CD spectrum. Based on the above evidences, compound **3** was characterized as (2*S*)-naringenin 4'-*O*-sulphate, previously obtained through metabolisation of 7-*O*-

methylnaringenin (sakuranetin) by *Cunninghamella elegans* NRRL 1392 [16], but reported now for the first time as a natural flavanone.

The following ten known compounds were also identified by comparison with published spectroscopic data and/or reference standards as succinic acid, ferulic acid, ferulic acid methyl ester [17], kaempferol 3-*O*-β-D-glucuronopyranoside [18], 5-hydroxyveratric acid [19], 5-hydroxyveratric acid methyl ester [20], naringenin [15], kaempferol [15], 4',7-di-*O*-methylkaempferol-3-*O*-sulphate [21], and gardenin A [4].

The total phenol, flavonoid and proanthocyanidin contents in methanol shoot extract of *T. africana* were assessed by using methods previously reported [22, 23]. Results presented in **Table 2** show a significant polyphenol content (over 150 mg GAE/g), a large amount of tannins (79.2 mg CE/g), and an appreciable flavonoid content (23.9 mg CE/g). Interestingly, *T. africana* contains a very high amount of proanthocyanidins compared to some other halophyte species belonging to the same family, such as *Reaumuria vermiculata* L. [24]. Moreover, the flavonoid content was significantly higher in *T. africana* shoot as compared to the halophyte *Limonium densiflorum* (Guss.) Kuntze [25] traditionally used as a medicinal plant. In fact, the high phenolic content of *T. africana* may be due to the hard conditions in which this species grows mainly salinity and aridity [26].

The high phenolic content of a plant extract is usually directly related to its antioxidant activity. For this reason, the oxygen radical absorbance capacity was determined by the ORAC assay [27]. The *T. africana* methanol extract exhibited significant antioxidant activity with ORAC value of 2.5 μmol TE/mg (**Table 2**), two fold higher than that reported for the methanol extract of the closely related species *Reaumuria vermiculata* (1.1 μmol TE/mg) [24]. Moreover, the antioxidant activity of *T. africana* shoot extract was also assessed in a cell-based assay which uses dichlorofluorescin-diacetate (DCFH-DA) as an indicator of reactive oxygen species (ROS), and allows the detection of both anti- and pro-oxidant properties [28]. The methanol extract of *T. africana* inhibited tert-butylhydroperoxide (tBH)-induced oxidation of DCFH with IC_{50} value of 3.3 μg/mL (**Table 2**). In comparison, the IC_{50} values of the standard antioxidant Trolox and quercetin were 0.1 μg/mL and

0.8 μg/mL, respectively. The inhibition of the tBH-induced oxidation of DCFH, is significantly higher for the *T. africana* shoot extract compared to *Zygophyllum album* L.f. methanol extract (IC_{50}= 103 μg/mL) [29].

Plant foods including fruits, vegetables and medicinal herbs are an excellent source of antioxidant molecules that effectively inhibit the inflammatory process by affecting different molecular targets [30, 31]. For this reason, the presence of phenolic compounds in *T. africana* extract prompted us to investigate its anti-inflammatory activity using RAW 264.7 macrophages which were stimulated to induce an overproduction of nitric oxide (NO). The methanol extract significantly inhibited NO release in a dose-dependent manner at concentrations ranging from 20 μg/mL (24.3%) to 160 μg/mL (53.5%) (**Fig. 2**). However, the positive control L-NAME (N_{ω}-Nitro-L-Arginine Methyl Ester) at 25 μg/mL inhibited NO release more efficiently (64.1%). The anti-inflammatory activity of the methanolic extract could be due, in part, to the presence of phenolic compounds. These data are in line with those reported in Oueslati et al. [32] where the methanol extract of *Suaeda fruticosa* Forssk. ex J.F.Gmel. showed the best anti-inflammatory activity compared to extracts of the same plant prepared with other solvents. Megdiche Ksouri et al. [29] also obtained similar results for *Zygophyllum album* showing that the methanol extract exhibited the strongest inhibition of NO release in a dose-dependent manner.

Plant phytochemicals have received increasing attention due to their specific cytotoxicity against cancer cells [33]. Phenolic compounds, which are powerful antioxidants, are considered as molecules with anti-inflammatory, antimutagenic and cytotoxic activities [34]. The cytotoxic activity of *T. africana* has not been reported in literature and the antioxidant activity of this species and its high phenolic content encouraged us to investigate its anti-proliferative capacity. Therefore, the cytotoxicity of *T. africana* was evaluated against the colon carcinoma (DLD-1) and lung carcinoma (A-549) cell lines as well as a healthy human skin fibroblast cell line (WS1). The methanol extract exhibited cytotoxic activity against A-549 cells with IC_{50} value of 34 μg/mL. On the other hand, no activity was noticed against DLD-1 and WS-1 cells. These data suggested that *T.*

africana has an influence on tumor cell viability and specifically targets the lung carcinoma cell line (A-549). Interestingly, the methanolic extract of the medicinal plant *Reaumuria vermiculata*, belonging to the same family as *T. africana*, was also reported to be cytotoxic against the tumor cell line A-549 with IC_{50} value of 99 µg/mL [24].

To the best of our knowledge, this is the first study on the antioxidant, anti-inflammatory and cytotoxic activities of *T. africana* shoot extract. The methanol extract contains a high amount of phenolic contents. It possesses antioxidant and anti-inflammatory activities and was found to be cytotoxic against the human lung carcinoma cell line (A-549). Three new sulphated flavonoids and ten known compounds were isolated and characterized from this extract. Glycosides containing sulphate residues have been identified previously in the plant kingdom, but the occurrence of sulphated flavan and flavan-4-ol derivatives is a very unusual finding. Particularly flavan-4-ols are rare compounds. Recently, Khalil et al. questioned about the real existence of apiforol (5,7,4'-trihydroxyflavan-4-ol), as well as other flavan-4-ols in *Sorghum* species, since apiforol has never been fully characterized from plant sources [35]. The isolation and complete characterization of compound **1** in *T. africana* could confirm the presence of flavan-4-ols as genuine natural products. Overall, this study provides new insights into the chemical constituents and bioactivities of *T. africana*.

Materials and Methods

General experimental procedures

Optical rotation values were determined on an AUTOPOL IV Automatic polarimeter (Rudolph Research Analytical) equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin–Elmer–Lambda Spectrophotometer. CD spectra were measured on a JASCO J-810 spectropolarimeter with a 0.1 cm cell in MeOH at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, bandwidth 2.0 nm. NMR experiments were performed on Bruker 250 Avance and Bruker DRX-600 spectrometers (Bruker BioSpin

GmBH) equipped with a Bruker 5 mm TCI CryoProbe at 300 K. Chemical shifts were expressed in δ (parts per million) referred to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD. Standard pulse sequences and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC experiments. MS and MS^n analyses (positive and negative mode) were obtained using a LCQ Advantage spectrometer (Thermo Finnigan) equipped with an ion trap analyzer and Xcalibur 3.1 software. Samples were injected into the spectrometer at a flow rate of 5 μL/min using an external syringe pump. HRESIMS experiments were achieved using a nano LC-MS/MS system, with a nanoAcquity UPLC module and a Q-TOF premier spectrometer equipped with a nanoelectrospray ion source (Waters), and provided with a lock-mass device to perform real-time calibration correction. Column chromatography was performed over Sephadex LH-20 (25-100 μm, Pharmacia Fine Chemicals) and SiO_2 (silica gel 60 SNAP cartridges, Biotage). HPLC was conducted on a Shimadzu instrument consisting of a LC-8A series pumping system, a RID10A refractive index detector, and a Rheodyne 7125 injector. Separations were performed on a C_{18} μ-Bondapak column (30 cm x 7.8 mm, 10 μm, Waters) at a flow rate of 2.0 mL/min. TLC was performed on precoated silicagel 60 F_{254} plates (Merck); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ (Sigma Aldrich) and NTS (Naturstoff reagent)-PEG (Polyethylene glycol 4000) solutions (Sigma Aldrich).

Chemicals

Folin–Ciocalteu reagent, sodium carbonate anhydrous (Na_2CO_3), sodium nitrite solution ($NaNO_2$), vanillin, aluminium chloride hexahydrate solution ($AlCl_3.6H_2O$), and gallic acid (purity > 97.5%) were purchased from Fluka. Sulphuric acid was obtained from Merck. Fluorescein sodium salt (FL), 20,70-dichlorofluorescin-diacetate (DCFH-DA), 20,70-dichlorofluorescin (DCFH), 20,70-dichlorofluorescein (DCF), tert-butyl hydroperoxide (*t*-BuOOH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), (+)-catechin (purity > 99%), quercetin (purity > 95%), and 2,20-azobis(2-amidino-propane) dihydrochloride (AAPH) were all purchased from Sigma–Aldrich. The solvents

were purchased from EMD. All other chemical reagents were purchased from Sigma–Aldrich or Alfa Aesar and used as received.

Plant material

T. africana shoots were collected in March 2012 from the region of Thelja (South West of Tunisia, GPS N 34° 20' E 20° 15', semi-arid climate). The plant was identified by Dr. Abderrazak Smaoui (CBBC, Borj-Cedria) and a voucher specimen was deposited at the Herbarium of the Laboratory of Extremophile Plants at the CBBC (T-TAF 126).

Extraction and isolation

The air-dried *T. africana* shoots were grinded to an uniform powder. Then, 400 g of powdered plant were extracted in a Soxhlet apparatus using solvents of increasing polarity (*n*-hexane, dichloromethane, methanol), overnight for each solvent. The solvents were evaporated under reduced pressure and the extracts freeze-dried. The methanol extract (30.6 g) was partitioned between *n*-butanol and water (1:1). The *n*-butanol fraction (4.7 g) was chromatographed over Sephadex LH-20 (3 cm x 60 cm) eluting with MeOH (flow rate 0.8 mL/min). 82 fractions of 10 mL were collected and combined after TLC analysis on silica gel plates with *n*-BuOH-AcOH-H_2O (60:15:25) and $CHCl_3$-MeOH-H_2O (70:30:3) into 13 fractions (A-M). Fraction D (600 mg, 230-270 mL) was separated through flash chromatography (25 g silica SNAP cartridges, flow rate 25 mL/min, 15 mL fractions) eluting with *n*-hexane-$CHCl_3$ (1:1), followed by $CHCl_3$ and increasing concentrations of MeOH in $CHCl_3$ (between 1% and 100%), to give succinic acid (21 mg, 765-810 mL, gradient $CHCl_3$-MeOH 8:2$\rightarrow$ MeOH) together with 4 subfractions (D_1-D_4). Fraction D_2 (40 mg, 300-510 mL, gradient $CHCl_3 \rightarrow CHCl_3$-MeOH 97:3) was purified by RP-HPLC with MeOH-H_2O (1:1) as eluent to give gardenin A (3 mg, t_R = 12 min). Fraction E (280.2 mg, 280-330 mL) was submitted to flash chromatography (10 g silica SNAP cartridges, flow rate 12 mL/min, 10 mL fractions) eluting with $CHCl_3$ followed by increasing concentrations of MeOH in $CHCl_3$ (between

1% and 100%) to give 3 subfractions (E_1-E_3). Fraction E_1 (138.9 mg, 50-60 mL, $CHCl_3$) was purified by RP-HPLC with MeOH-H_2O (2:3) to give ferulic acid (11.7 mg, t_R = 14 min), 5-hydroxyveratric acid methyl ester (14.6 mg, t_R = 23 min), and ferulic acid methyl ester (3.9 mg, t_R = 43 min). Fraction G (300 mg, 390-450 mL) was separated through flash chromatography (10 g silica SNAP cartridges, flow rate 12 mL/min, 10 mL fractions) eluting with $CHCl_3$ followed by increasing concentrations of MeOH in $CHCl_3$ (between 1% and 100%), to give kaempferol 3-*O*-β-D-glucuronopyranoside (11.7 mg, 260-290 mL, $CHCl_3$-MeOH 1:1) together with 3 subfractions (G_1-G_3); fraction G_3 (61.4 mg, 200-230 mL, gradient $CHCl_3$-MeOH 3:2→1:1) was purified by RP-HPLC with MeOH-H_2O (2:3) as eluent to give 5-hydroxyveratric acid (1 mg, t_R = 5 min). Fractions I (89.7 mg, 490-530 mL) and K (68.5 mg, 650-720 mL) were separately purified by RP-HPLC with MeOH-H_2O (3:2) as eluent to give naringenin (4.6 mg, t_R = 9 min) from fraction I, and kaempferol (2 mg, t_R = 14 min) and 4',7-di-*O*-methylkaempferol-3-*O*-sulphate (1.5 mg, t_R = 7 min) from fraction K. Fraction J (104.5 mg, 540-640 mL) was subjected to RP-HPLC using MeOH-H_2O (2.5:7.5) to yield compounds **2** (1.4 mg, t_R = 10 min) and **3** (4.2 mg, t_R = 13 min). Fraction L (122.0 mg, 730-820 mL) was purified through RP-HPLC using MeOH-H_2O (3.5:6.5) to yield compound **1** (5.5 mg, t_R = 6 min). All the compounds met the criteria of ≥95% purity, as inferred by HPLC and NMR analyses.

(2S,4R)-4,5,7,4'-Tetrahydroxyflavan-4-ol 5,7-di-O-sulphate (**1**): red-brownish amorphous powder; $[\alpha]_D^{25}$ +22.7 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε): 297 (3.69); CD $[\theta]_{25}$ (*c* 0.1, MeOH) + 3200 (230 nm), + 3000 (275 nm); ^{1}H and ^{13}C NMR data, see **Table 1**; HRESIMS: *m/z* 454.9733 $[M-H]^-$ (calcd. for $C_{15}H_{13}NaO_{11}S_2$, *m/z* 454.9719); MS/MS *m/z* 335.02 $[(M-120)-H]^-$, 255.07 $[(M-120-80)-H]^-$, 152.01 $[(M-120-80-103)-H]^-$.

(2S)-5,7,4'-Trihydroxyflavan 7-O-sulphate (**2**): red-brownish amorphous powder; $[\alpha]_D^{25}$ -8.1 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε): 287 (3.74); CD $[\theta]_{25}$ (*c* 0.1, MeOH) - 3300 (280 nm); ^{1}H and ^{13}C

NMR data, see **Table 1**; HRESIMS: *m/z* 337.0235 $[M-H]^-$ (calcd. for $C_{15}H_{13}O_7S$, *m/z* 337.0382); MS/MS *m/z* 257.14 $[(M-80)-H]^-$, 217.06 $[(M-120)-H]^-$, 137.15 $[(M-120-80)-H]^-$.

(2S)-Naringenin 4'-O-sulphate (**3**): yellow amorphous powder; $[\alpha]_D^{25}$ -45 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε): 287 (3.90), 327 (4.05); CD $[\theta]_{25}$ (*c* 0.1, MeOH) - 3250 (282 nm), + 2850 (330 nm); ^1H and ^{13}C NMR data, see **Table 1**; HRESIMS: *m/z* 351.0189 $[M-H]^-$ (calcd. for $C_{15}H_{11}O_8S$, *m/z* 351.0175); ESIMS: *m/z* 351 $[M-H]^-$, 271 $[(M-80)-H]^-$.

Biological activity assays

Total polyphenolic content (TPC)

Total phenolic content was determined by the Folin–Ciocalteu colorimetric method slightly modified by Dewanto et al. [22] using gallic acid as standard. An aliquot (0.125 mL) of tenfold diluted extract, dissolved in methanol (0.1g/mL), was added to 0.5 mL of distilled water and 0.125 mL of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before addition of 1.25 mL of Na_2CO_3 (7%). The solution was then adjusted with distilled water to a final volume of 3 mL, mixed thoroughly, and held for 90 min at ambient temperature. After incubation in dark, the absorbance at 760 nm was recorded. Total phenolic content was expressed as mg GAE/g dry extract (mg GAE/ g) through the calibration curve with gallic acid. Triplicate measurements were carried out for all samples.

Total flavonoid content

Total flavonoids were measured by a colorimetric assay according to Dewanto et al. [22]. An aliquot (0.125 mL) of twofold diluted methanol extract, dissolved in methanol (0.1g/mL), or standard solution of (+)-catechin, was added to a 75 µl of $NaNO_2$ (7%) solution, and mixed for 6 min, before adding 0.15 mL $AlCl_3$ (10%). After 5 min, 0.5 mL of 1 M NaOH was added. The final volume was adjusted to 2.5 mL with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm against the same mixture, without the sample, as a blank. Total

flavonoid content was expressed as mg catechin/g dry extract (mg CE /g), through the calibration curve of (+)-catechin. The calibration curve range was 0–400 µg/mL (R^2 = 0.99). All samples were analyzed in triplicates.

Total condensed tannins

The analysis of condensed tannins (proanthocyanidins) was carried out according to the method of Sun et al. [23]. 3 mL of 4% methanolic vanillin solution and 1.5 mL of concentrated H_2SO_4 were added to 0.05 mL of samples dissolved in methanol (0.1 g/mL). The mixture was allowed to stand for 15 min, and the absorbance was measured at 500 nm. The amount of total condensed tannins was expressed as mg catechin/g dry extract (mg CE/g), through the calibration curve of (+)-catechin. All samples were analyzed in triplicates.

ORAC assay

The procedure was modified from the method described by Ou et al. [36]. Briefly, the ORAC assay was carried out in black round bottom 96-well microplates (Costar) on a Fluoroskan Ascent FlTM plate reader (Labsystems). Trolox was used as the control standard. Four concentrations of Trolox were used (1.6, 3.1, 6.5 and 12.5 µM) in quadruplicate, and a gradient of 16 concentrations of the samples, dissolved in DMSO, was prepared in three replications. The experiment was conducted at 37.5 °C and pH 7.4, with a blank sample in parallel. The fluorimeter was programmed to record the fluorescence of fluorescein every 30 s after addition of 2,2'-azobis-(2-amidinopropane)-dihydrochloride (AAPH). The results were calculated by comparing the net areas under the fluorescein decay curves between the blank and the samples. ORAC values were expressed in micromoles of Trolox equivalents (TE) per milligram (µmol TE/mg).

2',7'-dichlorofluorescin-diacetate (DCFH-DA) assay

Antioxidant activity was evaluated using the DCFH-DA assay as described by Legault et al. [37], with some modifications. Briefly, human skin fibroblast cells were plated in 96 microwell plates at 10,000 cells per well and incubated for 24 h at 37 °C and 5% CO_2. The cells were washed with 150 µL Hank's balanced salt solution (HBSS) at pH 7.4 and incubated for 30 min with 100 µL HBSS (pH 7.4) containing 5 µM DCFH-DA (Sigma-Aldrich). The cells were then washed again with 150 µL HBSS. To assess antioxidant activity, the cells were incubated with a growing concentration (ranging from 6.25 to 200 µg/mL) of *T. africana* methanol extract dissolved in DMSO, Trolox or quercetin (also dissolved in DMSO), in the absence or presence of 200 µM *t*BH. The samples were analyzed in triplicates. Fluorescence was measured after 1 and 4 h on an automated 96-well plate reader (Fluoroskan Ascent FLTM, Labsystems) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. IC_{50} were calculated using the logarithmic regression of the dose–response curve after subtraction of both blank and intrinsic sample fluorescence values. In all cases, the regression coefficients (R^2) were greater than 0.95. IC_{50} are the means ± standard deviations of three determinations.

Cell culture

The human lung carcinoma A-549 (ATCC CCL-185), colon adenocarcinoma DLD-1 (ATCC CCL-221), normal skin fibroblast (WS-1) and murine macrophage RAW 264.7 (ATCC TIB-71) cell lines were obtained from the American Type Culture Collection (ATCC). The A-549, DLD-1 lines were grown in Minimum Essential Medium with Earle's salts, while the RAW 264.7 cell line was grown in Dulbecco's modified Eagle's medium (Mediatech Cellgro). Both media were supplemented with 10% fetal calf serum (Hyclone), solution of vitamins (1×), sodium pyruvate (1×), non-essential amino acids (1×), penicillin (100 IU) and streptomycin (100 µg/mL) (Mediatech Cellgro). Cells were cultured in a humidified atmosphere at 37 ◦C in 5% CO_2.

Anti-inflammatory activity assay

To investigate the anti-inflammatory activity of *T. africana* methanol extract, nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells was examined. Exponentially growing cells were plated in 24-well microplates (BD Falcon) at a density of 2×10^5 cells per well in 400 µl of culture medium and were allowed to adhere overnight. Cells were then treated or not with positive control L-NAME or increasing concentrations of methanol extract dissolved in DMSO and incubated at 37 °C, 5% CO_2 for 24 h. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cells were then stimulated with 100 µg/mL LPS prepared from *Escherichia coli*. After 24 h, cell-free supernatants were collected and nitrite production was measured using the modified method of Green et al. [38]. Griess reagent (50 µL of 1% sulphanilamide and 50 µL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) was added in equal volume (100 µL) to cell supernatant and incubated at room temperature for 20 min. The absorbance at 540 nm was then measured using an automated 96-well Varioskan Ascent plate reader (Thermo Electron) and nitrite was quantified by comparison with a $NaNO_2$ standard curve. Triplicate measurements were carried out for all samples.

Cytotoxicity assay

Exponentially growing cells were plated at a density of 5×10^3 cells per well, in 96-well microplates (Costar, Corning Inc.) into 100 µL of culture medium and were allowed to adhere for 16 h at 37 °C under 5% CO_2 before treatment. Then, 100 µL of increasing concentrations of methanol extract dissolved in DMSO were added. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. The cells were incubated for 48 h in the presence or absence of the extract. Cytotoxicity was assessed using the resazurin reduction test as described by O'Brien et al. [39]. Fluorescence was measured using an automated 96-well Fluoroskan Ascent Fl™ plate reader (Thermo-Labsystems) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Cytotoxic activity was expressed as the concentration of extract inhibiting cell growth by 50% (IC_{50}). Etoposide was used as positive control (IC_{50} A-549 cells, 1.4 µM; IC_{50}

DLD-1 cells, 1.4 µM; IC_{50} WS-1 cells, 8 µM). Triplicate measurements were carried out for all samples.

Statistical analysis

For each spectrophotometric test three independent experiments were carried out. ANOVA procedure was applied to test the difference between the solvent and standards. *P* values of 0.05 or less were considered as statistically significant. IC_{50} values were calculated with Excel (2010).

Supporting information

NMR spectra of compounds **1-3** and data for IC_{50} determination of cytotoxic activity are available as supporting information. This material is available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Conflict of Interest

The authors declare no conflict of interest.

References

[1] *Khabtane A*, *Rahmoune C*. Effet du biotope sur la diversité floristique et le polymorphisme phénotypique des groupements à *Tamarix africana* Poir. Dans les zones arides de la région de Khenchela (Est Algérien). Revue JAEID 2012; 106: 123-137.

[2] *Ksouri R, Megdiche Ksouri W, Jallali I, Debez A, Magné C, Hiroko I, Abdelly C*. Medicinal halophytes: potent source of health promoting biomolecules with medical, nutraceutical and food applications. Critical Rev Biotechnol 2012; 32: 289-326

[3] *Sharma V, Bhardwaj U, Sharma S, Sharma S*. Medicinal plants: need for sustainable exploitation (with special reference to Himachal Pradesh). J Pharm Res 2012; 5: 4313-4317

[4] *Parmar VS, Bisht KS, Sharma SK, Jain R, Tanya P, Singh S, Simonsen O, Boll PM*. Highly oxygenated bioactive flavones from *Tamarix*. Phytochemistry 1994; 36: 507-511

[5] *Ahmet A, Yurdanur A*. A new analogue of fatty alcohol from *Tamarix hampeana* L. Nat Prod Res 2010; 24: 34-39

[6] *Sharma SK, Parmar VS*. Novel constituents of *Tamarix* species. J Sci Ind Res 1998; 57: 873-890

[7] *Saïdana D, Mahjoub MA, Boussaada O, Chriaa J, Chéraif I, Daami M, Mighri Z, Helal AN*. Chemical composition and antimicrobial activity of volatile compounds of *Tamarix boveana* (Tamaricaceae). Microbiol Res 2008; 163: 445-455

[8] *Nawwar MAM, Buddrus J, Bauer HL*. Dimeric phenolic constituents from the roots of *Tamarix nilotica*. Phytochemistry 1982; 21: 1755-175

[9] *Saleh NAM, El-Sissi HI*. A rhamnetin glucuronide trisulphate from the leaves of *Tamarix aphylla*. Phytochemistry 1975; 14: 312-314

[10] *Younos C, Soulimani R, Seddiqi N, Baburi O, Dicho A*. Étude ethnobotanique et historique des tamaris (*Tamarix* sp., Tamaricaceae) et leurs usages actuels en Afghanistan. Phytothérapie 2005; 3: 248-251

[11] *Falleh H, Oueslati S, Guyot S, Ben Dali A, Magné C, Abdelly C, Ksouri R*. LC/ESI-MS/MS characterisation of procyanidins and propelargonidins responsible for the strong antioxidant activity of the edible halophyte *Mesembryanthemum edule* L. Food Chem 2011; 127: 1732-1738

[12] *Zhao Z, Ruan J, Jin J, Zou J, Zhou D, Fang W, Zeng F*. Flavan-4-ol glycosydes from the rhizomes of *Abacopteris penangiana*. J Nat Prod 2006; 69: 265-268

[13] *Slade D, Ferreira D, Marais JPJ*. Circular dichroism, a powerful tool for the assessment of absolute configuration of flavonoids. Phytochemistry 2005; 66: 2177-2215

[14] *Okamoto A, Ozawa T, Imagawa H, Arai Y.* Flavans from the pith of sago palm. Agric Biol Chem 1986; 50: 1655-1656

[15] *Agrawal PK*. Carbon-13 NMR of flavonoids. Amsterdam: Elsevier; 1989: 102

[16] *Ibrahim AR, Galal AM, Ahmed MS, Mossa GS*. *O*-demethylation and sulfation of 7-methoxylated flavanones by *Cunninghamella elegans*. Chem Pharm Bull 2003; 51: 203-206

[17] *Abdel-Mogib M, Basaif SA, Al-Garni SM*. Antimicrobial activity and chemical constituents of leaf extracts of *Tamarix aphylla*. Alexandria J Pharm Sci 2001; 15: 121-123

[18] *Merfort I, Wendisch D.* Flavonolglucuronide from the flowers of *Arnica montana*. Planta Med 1988: 247-250

[19] *Pettit GR, Singh SB.* Antineoplastic agents. Isolation, structure, and synthesis of combretastatin A-2, A-3, and B-2. Canad J Chem 1987; 65: 2390-2396

[20] *Tanaka M, Ikeya Y, Mitsuhashi H, Maruno M, Wakamatsu T*. Total synthesis of the metabolites of schizandrin. Tetrahedron 1995; 51: 11703-11724

[21] *Nawwar MAM, Souleman AMA, Buddrus J, Linscheid M.* Flavonoids of the flowers of *Tamarix nilotica*. Phytochemistry 1984; 23: 2347-2349

[22] *Dewanto V, Wu X, Adom KK, Liu RH*. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem 2002; 50: 3010-3014

[23] *Sun B, Richardo-da-Silvia M, Spranger I*. Critical factors of vanillin assay for catechins and proanthocyanidins. J Agric Food Chem 1998, 46: 4267-4274

[24] *Karker M, Falleh H, Msaada K, Smaoui A, Abdelly C, Legault J, Ksouri R.* Antioxidant, anti-inflammatory and anticancer activities of the medicinal halophyte *Reaumuria vermiculata.* Excli J 2016; 15: 297-307

[25] *Medini F, Bourgou S, Girard-Lalancette K, Snoussi M, Mkadmini K, Cot I, Abdelly C, Legault J, Ksouri R*. Phytochemical analysis, antioxidant, anti-inflammatory, and anticancer activities of the halophyte *Limonium densiflorum* extracts on human cell lines and murine macrophages. S Afr J Bot 2015; 99: 158-164

[26] *Sanchez-Rodrıguez E, Moreno DA, Ferreres F, Rubio-Wilhelmi MM, Ruiz JM*. Differential responses of five cherry tomato varieties to water stress: changes on phenolic metabolites and related enzymes. Phytochemistry 2011; 2: 723-729

[27] *Awika JM, Rooney LW, Wu X, Prior RL, Zevallos LC*. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and *Sorghum products*. J Agric Food Chem 2003; 51: 6657-6662

[28] *Girard-Lalancette K, Pichette A, Legault J.* Sensitive cell-based assay using DCFH oxidation for the determination of pro- and antioxidant properties of compounds and mixtures: analysis of fruit and vegetable juices. Food Chem 2009; 115: 720-726

[29] *Megdiche Ksouri W, Medini F, Mkadmini K, Legault J, Abdelly C, Ksouri R*. LC–ESI TOF–MS identification of bioactive secondary metabolites involved in the antioxidant, anti-inflammatory and anticancer activities of the edible halophyte *Zygophyllum album* Desf. Food Chem 2013; 139: 1073-1080

[30] *Tsai PJ, Tsai TH, Yu CH, Ho SC.* Evaluation of NO suppressing activity of several Mediterranean culinary spices. Food Chem Toxicol 2007; 45: 440-447

[31] *Taira J, Nanbu H, Ueda K*. Nitric oxide-scavenging compounds in *Agrimonia pilosa* Ledeb on LPS-induced RAW264.7 macrophages. Food Chem. 2009; 115: 1221-1227

[32] *Oueslati S, Ksouri R, Falleh H, Pichette A, Abdelly C, Legault J.* Phenolic content, antioxidant, anti-inflammatory and anticancer activities of the edible halophyte *Suaeda fruticosa* Forssk. Food Chem 2012; 132: 943-947

[33] *Nemeikaite-Ceniene A, Imbrasaite A, Sergediene E, Cenas N*. Quantitative structure–activity relationships in prooxidant cytotoxicity of polyphenols: role of potential of phenoxyl radical/phenol redox couple. Arch Biochem Biophys 2005; 441: 182-190

[34] *Vuorela S, Kreander K, Karonen M, Nieminen R, Hämäläinen M, Galkin A, Laitinen L, Salminen JP, Moilanen E, Pihlaja K, Vuorela H, Vuorela P, Heinonen M*. Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. J Agric Food Chem 2005, *53*, 5922-5931

[35] *Khalil A, Baltenweck-Guyot R, Ocampo-Torres R, Albrecht P.* Retrodihydrochalcones in *Sorghum* species: key intermediates in the biosynthesis of 3-deoxyanthocyanidins? Phytochem Lett 2012; 5: 174-176

[36] *Ou B, Hampsch-Woodill M, Prior RL.* Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem 2001; 49: 4619-4626

[37] *Legault J, Dahl W, Debiton E, Pichette A, Madelmont JC*. Antitumor activity of balsam fir oil: Production of reactive oxygen species induced by alpha-humulene as possible mechanism of action. Planta Med 2003; 69: 402-407

[38] *Green SJ, Meltzer MS, Hibbs Jr JB, Nacy CA*. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J Immunol 1990; 144: 278-283

[39] *O'Brien J, Wilson I, Orton T, Pognan F.* Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000; 267: 5421-5426

Figure legends:

Fig. 1 Chemical structures of compounds **1-3** isolated from *T. africana* shoot methanol extract

Fig. 2 Effect of *T. africana* shoot methanol extract on NO overproduction in LPS-stimulated RAW 264.7 macrophages.

Table 1 ^{1}H and ^{13}C-NMR data of compounds **1-3** (CD_3OD, 600 MHz, *J* in Hz)

position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	5.20 dd (12.9, 2.0)	74.6	4.88[a]	78.9	4.43 dd (12.8, 3.2)	79.4
3a	2.13 br dd (16.0, 2.0)	38.4	2.13 m	30.0	3.07 dd (17.2, 12.8)	43.9
3b	1.98 br dd (16.0, 12.9)		1.95 m		2.75 dd (17.2, 3.2)	
4a	5.17 br s	60.0	2.93 br dd (13.0, 1.0)	21.0		196.7
4b			2.80 m			
5		155.0		157.0		165.6
6	7.03 d (2.0)	109.4	6.65 d (1.8)	102.3	5.88[a]	96.7
7		153.6		152.0		170.3
8	6.82[a]	107.6	6.14 d (1.8)	101.0	5.88[a]	96.7
9		158.0		153.0		164.6
10		116.2		108.5		103.0
1'		132.9		134.6		136.9
2'/6'	7.30 d (8.6)	128.6	7.24 d (8.5)	128.5	7.48 d (8.0)	128.3
3'/5'	6.82[a]	115.9	6.80 d (8.5)	116.1	7.36 d (8.0)	122.6
4'		158.2		157.0		154.2

Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

[a] Overlapped signal.

Table 2 Total polyphenol, flavonoid and condensed tannin contents, ORAC (oxygen radical absorbance capacity) values, and antioxidant activity of *T. africana* methanol extract and standards. Values (means of three replications) of the same column followed by different letters are significantly different at $P < 0.05$.

	Polyphenols (mg GAE/g)	Flavonoids (mg CE/g)	Tannins (mg CE/g)	ORAC value (µmol Trolox/mg)	Antioxidant activity (IC_{50} in µg/mL)
Methanol extract	151.1± 10.9	23.9± 2.9	79.2± 8.6	2.5±0.2[a]	3.3±0.4[c]
Trolox				4.1±0.4[b]	0.1±0.0[a]
Quercetin				21.3±1.4[c]	0.8±0.2[b]