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Aphyllin, the first isoferulic acid glycoside and other phenolics from *Tamarix aphylla* flowers

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The first glycosylated isoferulic acid, isoferulic acid 3-*O*-β-glucopyranoside, together with the new phenolics, tamarixetin 3,3′-di-sodium sulphate and dehydrodigallic acid dimetyl ester have been characterized from a flower extract of *Tamarix aphylla*. The structures were established on the basis of spectral data. The extract exhibited a distinct radical scavenging effect and to improve the viability of human keratinocytes (HaCaT cells). Also, the known isoferulic acid and ferulic acid which have been determined to be the major components of the investigated extract by HPLC/ESI mass spectrometric screening have been separated, characterized and evaluated as active antioxidants and as cell activity stimulating agents as well.

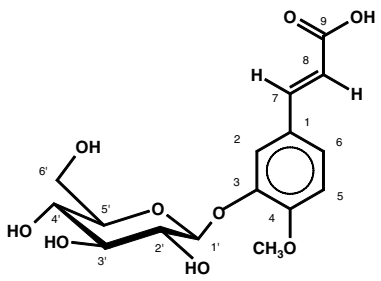
1. Introduction

The majority of plant phenolics are known to be powerful antioxidants either by direct scavenging of reactive oxygen and nitrogen species or by acting as chain-breaking peroxyl radical scavengers (Korkina and Afanas'ev 1997; Kostyuk et al. 2008). They are able therefore, to act against free radical-mediated UV-induced cellular death also in cultures of human keratinocytes (F'guyer and Mukhtar 2003; Abou Zaid et al. 2007). Among Egyptian plants rich in phenolics, the genus *Tamarix* (Tamaricaceae) comprises six species, including *Tamarix aphylla* L. (synonym: *Thuja aphylla* L., *Tamarix orientalis* Forssk, *Tamarix articulate* Vahl) which was reported to be capable on synthesizing and accumulating high percent of phenolics (Nawwar et al. 1975; El Ansari et al. 1976; Souleman et al. 1991; Merfort et al. 1992; Nawwar et al. 1994a, b). However, the phenolics of the flowers of this plant (El Ansari et al. 1976; Nawwar et al. 1975) remained partially unexplored. In the present study, the aqueous methanol extract of the flowers has been investigated. We report the isolation and structural determination of two new phenyl propanoid derivatives, namely, isoferulic acid 3-*O*-glucopyranoside (**1**); tamarixetin 3,3′-di-sodium sulphate (**2**) and the new phenolic acid ester, dehydrodigallic acid dimetyl ester (**3**) together with the two major known constituents, isoferulic acid (**4**) (Nawwar et al., 1982) and ferulic acid (**5**) (Soulemanet al. 1998). Besides, These flowers have not been subjected before to any biological assessment, therefore, we investigate in the present work the effects of the aqueous methanol extract and the two major known constituents isoferulic acid and ferulic acid for their radical scavenging capacity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method (Sievers et al. 2002; Brand et al. 1995) as well as for their cell viability changing activity using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay (Mosmann 1983). As cellular test model we used the HaCaT (Human adult low Calcium high Temperature) keratinocyte cell line, an immortalized and spontaneously transformed non-tumorigenic human epidermal cell line. It does not require a 3T3 feeder layer and has the capacity for normal differentiation *in vitro* (Boukamp et al. 1988). This cell line offers the best *in vitro* model for the study of regulatory mechanisms involved in the differentiation of human epidermal cells.

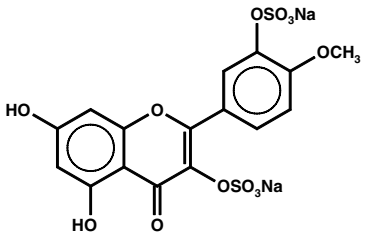
2. Investigations, results and discussion

2.1. Isolation and structure elucidation

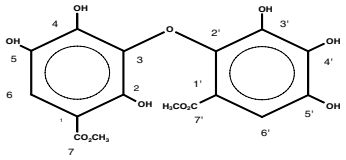
By two dimensional paper chromatography and HPLC/ESI-MS it was shown that the aqueous methanol extract of the flowers of *Tamarix aphylla* contains a complex phenolic mixture. To fractionate this mixture it was exhaustively extracted by acetone to yield an acetone fraction



Isoferulic acid 3-*O*-β-$^4C_1$-glucopyranoside (**1**)



Tamarixetin 3,3′-di-sodium sulphate (**2**)



Dehydrodigallic acid dimethyl ester (**3**)

and a residue. Following adsorption column chromatography, two compounds (**1** and **2**) were isolated from the residue, while three compounds (**3**, **4** and **5**) were isolated from the acetone fraction. On the other hand, a direct extraction of a flower sample by acetone followed by -ve HPLC/ESI-MS analysis (Nawwar et al. 1997) of the obtained acetone extract proved that the methyl ester (**3**) is not an artifact, whereby a negative ion $[M-H]^-$ of $m/z = 355$ corresponding to the molecular weight (356) of the ester was recognized in the received spectrum. Same result was received by -ve HPLC/ESI-MS analysis of the parent aqueous methanol extract.

Compound **1** was separated as a faint yellow amorphous powder and appeared on paper chromatograms, under UV light as blue spot which turned light blue on fuming with ammonia vapor. It presented a UV absorption spectrum in MeOH (λ_{max}: 235, 292, 309) which were reminiscent of phenyl propene system. Normal acid hydrolysis of **1** (2 N aqueous HCl, 3 h, 100 °C) yielded glucose (CoPC) and isoferulic acid (CoPC, mauve spot turning yellowish orange when fumed with ammonia, UV and ^{1}H NMR spectral data). The compound was also hydrolysed after being incubated with β-glucosidase enzyme (lyophilized chromatographically pure, salts free enzyme from Almond, BDH) for 24 h, at 37 °C to yield isoferulic acid. These data suggested that **1** is built up from an isoferulic acid moiety to which a glucoside moiety is attached at its C-3 and not via its carboxyl group. Connection of the glucose moiety through esterification of the carboxyl group of isoferulic acid would give an ester possessing the same color on PC under UV light as the free isoferulic acid itself. Compound **1** had the molecular formula $C_{16}H_{20}O_9$ as determined by HRESIFTMS ($[M-H]^-$: 355.1112, calc.: 355.1101). The suggested structure of **1** was then confirmed by NMR spectroscopy (room temperature, DMSO-d_6). From the ^{13}C NMR spectra, the presence of the glucose moiety followed from the signal at δ 101.6 ppm (Table 1) attributable to an anomeric glucose carbon adapting a β-configuration (Breitmaier and Voelter 1978). Attachment of the glucose moiety through the isoferulic hydroxyl group at C-3 was indicated by the downfield shift of the glucose C-1 signal (Δ ppm = 4.5) in comparison with the chemical shift of the corresponding carbon in free β-glucose (Kalinowski et al. 1984). The remaining five glucose carbons were found resonating in the sugar region between δ 77.3 and 60.9 ppm. The chemical shift values of all sugar carbons confirmed the pyranose form of the glucose moiety. That the sugar moiety must be attached to position 3 of isoferulic acid followed from the upfield shift of the signal of C-3 while the corresponding ortho and para carbon signals were shifted down field (all in comparison with the signals of the corresponding carbons in free isoferulic acid, see Experimental). Similar shifts are well known from the phenyl propanoid derivatives, flavonoid glycosides (Merkham et al. 1978). Assignments of the ^{13}C signals in this spectrum have been confirmed through DEPT and ^{1}H-^{13}C coupled spectra. Final

Table 1: ^{13}C NMR, DEPT, ^{1}H-^{13}C coupling constants and HMBC data of isoferulic acid 3-*O*-glucoside (1) and ^{13}C NMR data of isoferulic acid (4), tamarixetin, tamarixetin-3,3′-di-sodium sulphate (2), dehydrodigallic acid and dehydrodigallic acid dimethyl ester (3)

	Isoferulic acid	**1**		Tamarixetin	**2**	Dehydrodigallic Acid	**3**
	δ_c	δ_c, multiplicity, $^1J_{(C-H)}$, HMBC		δ_c	δ_c	δ_c	δ_c
1	127.68	127.6 s				120.6	121.2
2	113.99	116.1 d (165 Hz)	4,6,7,2,1#,3#	146.2	157.2	111.3	112.2
3	147.27	146.7 s		136.0	132.9	148.0	148.0
4	150.09	151.8 s		175.9	177.4	139.6	139.7
5	111.77	113.3 d (163 Hz)	1,3,4#,6#	160.8	160.0	146.1	146.4
6	121.07	124.9 d (165 Hz)	2,4,6,7,1#,5#	93.6	93.9	107.1	107.3
7	144.33	145.3 d (154 Hz)	2,6,9	163.9	163.3	168.2	166.9
8	116.36	115.4 d (156 Hz)	1,9	98.2	99.0		
9				156.2	157.3		
10				103.5	104.4		
1′		101.6 d (162 Hz)	3	123.2	123.4	115.7	115.7
2′		73.2 d (145 Hz)		114.8	121.1	136.6	136.6
3′		77.4 d (140 Hz)		146.2	141.0	140.0	140.4
4′		69.9 d(142 Hz)		149.1	151.7	139.7	139.9
5′		76.2 d 146 Hz)		111.5	111.3	143.0	143.3
6′		60.9 t (141 Hz)		119.4	127.8	109.0	109.7
7′						166.9	165.7
C=O	168.07	168.1 s					
OMe	55.44	56.0 q (146 Hz)	4	55.9	56.2		
Meester						52.0	51.9

: weak

confirmation was achieved through the direct correlation observation in the HSQC and HMBC spectra which allowed unambiguous assignments of the protonated carbons and enabled determination of site of attachment of the two moieties in the molecule of **1** (Table 1). Among the ^3J correlations recognized in the HMBC spectrum of **1**, one was found correlating the methoxyl proton signal at δ 3.72 to the aromatic carbon signal of C-4 at δ 151.8 and another correlated the anomeric glucose proton signal at δ 4.86 to the aromatic carbon signal of C-3 at δ 146.7. The ^1H NMR spectrum of **1** was also in accordance with the achieved structure. The glucose anomeric proton gave rise to a doublet ($J = 8$ Hz) signal, the position of which (δ 4.86) indicated the attachment of the anomeric carbon to the phenolic hydroxyl group of isoferulic acid. The lowfield location (δ ppm 7.59) of the meta coupled ($\Delta\nu_{1/2} = 4$ Hz) H-2 aromatic proton, in comparison with the corresponding signal in the spectrum of free isoferulic acid (see Experimental), is indicative of an ortho-substitution at OH-3. This spectrum also proved that the conformation of the glucose moiety is $^4C_1$. This follows from the β-configuration discussed above. These and the above given data finally confirmed the structure of compound **1** to be isoferulic acid 3-*O*-β-$^4C_1$-glucopyranoside, a new phenyl propanoid derivative, which has not previously been reported to occur in nature.

Compound **2**, is an off-white amorphous powder. It exhibited chromatographic properties, electrophoretic anionic character and UV maxima in MeOH and after addition of diagnostic shift reagents (see Experimental), which suggested its structure to be 3,3′,4′-trisubstituted quercetin (Harborne and Williams 1975; Mabry et al. 1970). On normal acid hydrolysis (2 N aqueous HCl, 15 min, 100 °C) (Barron et al. 1988), **2** yielded a flavonol aglycone, which precipitated from the aqueous hydrolysate, filtered off and identified by CoPC, UV, EI-MS and ^1H NMR spectral analysis as quercetin 4′-methyl ether, tamarixetin (Hussein 1992), but no sugar was released. On controlled acid hydrolysis (0.05 N aqueous HCl, 15 min, 100 °C, traced by paper chromatography each 3 min) **2** yielded two intermediates **2a** and **2b** which were separated by preparative PC. The chromatographic and electrophoretic properties, UV and ^1H NMR spectral data of **2a** were closely similar to those reported for tamarixetin 3-sulphate (Barakat 1998). The data obtained through the same analytical techniques (see Experimental) led to the identification of **2b** as tamarixetin 3′-sulphate. All hydrolysates gave a precipitate with aqueous $BaCl_2$ solution, thus proving the presence of sulphate group/s in intermediates (**2a** and **2b**). The aqueous solution of **2** failed to give any precipitate with sodium hexanitrocobaltate specific for potassium ion, but the presence of sodium ion in this aqueous solution was confirmed by flame atomic absorption (sodium spectral line detected at 589 nm). Compound **2** showed, in its -ve ESI-MS spectrum a molecular ion $[M-Na]^-$ at $m/z = 497$, and significant ions at $m/z = 417$, 395 and 315, attributable to $[M-SO_3Na]^-$, $[417 + H-Na]^-$ and $[417 + H-SO_3Na]^-$ ions, respectively, while on +pv ESI-MS analysis, it exhibited a molecular ion $[M + H]^+$ at $m/z = 521$, together with an $[M + 23]^+$ ion at m/z 543, corresponding to the molecular formula $C_{16}H_{10}O_{13}S_2Na_2$. The ^1H NMR spectrum of **2** (DMSO-d_6, room temp.) proved substitution at OH-3′ of the tamarixetin moiety. This followed from the lowfield location (δ 7.89 ppm) of the resonance of the H-2′ proton (broad singlet, ($\Delta\nu_{1/2} = 4$ Hz), in comparison with the corresponding signal in the spectrum of the aglycone tamarixetin. Other signals in the spectrum possessed chemical shifts and J values similar to those of tamarixetin and tamarixetin 3-sulphate. In the ^{13}C spectrum of **2** (Table 1) the attachment of the sulphate substituents to C-3 and C-3′ of tamarixetin followed from the upfield shift of the resonances of these carbons and the downfield shifts of the resonances of their ortho- and para- located carbons (see Experimental), all in comparison with the corresponding carbon resonances in the spectrum of free tamarixetin. Similar shifts have been reported before (Barron and Ibrahim 1988; Nawwar et al. 1981). Other resonances in this spectrum exhibited chemical shifts which agreed well with the achieved structure of **2** as tamarixetin 3,3′-di-sodium sulphate, a natural product which has not been reported before to occur in nature.

Compound **3**, was isolated as an off-white powder which exhibited chromatographic behavior and UV spectral data with an intense peak at $\lambda_{max} = 274$ nm, in MeOH similar to those given by gallic acid derivatives. Its EI-M spectrum revealed an $[M]^+$ ion at $m/z = 366$. Normal acid hydrolysis (2N aqueous HCL, 3 h, 100 °C) yielded dehydrodigallic acid (CoPC, ^1H NMR and EI-MS analysis). The small difference in molecular weight between that of compound **3** and of free dehydrodigallic acid, together with the result of acid hydrolysis suggested the presence of di-methyl ester groups in the molecule of **3**. The ^1H NMR spectrum (DMSO-d_6, room temp.) of **3**, showed five distinct proton signals, three of which were located in the aromatic region and were arranged in a pattern closely similar to that shown in the spectrum of free dehyrodigallic acid. These aromatic proton resonances were assigned as follows: δ (ppm), 7.14 (d, $J = 2$ Hz, H-2); 7.02 (s, H-8) and 6.65 (d, $J = 2$ Hz, H-8b). The remaining two singlet resonances at δ ppm, 3.65 and 3.55, were obviously due to the protons of the two methyl ester groups, thus proving the conclusion that compound **3** is the dimethyl ester of dehydrodigallic acid. The ^{13}C NMR spectrum finally confirmed the suggested structure of dehydrodigallic acid dimethyl ester and revealed 14 definite sp2 carbon resonances together with two sp3 methyl ester carbon resonances as well. Assignments of these resonances were aided by comparison with the previously reported ^{13}C NMR data of free dehydrodigallic acid (Nawwar et al. 1982). The above given data finally confirmed the identity of **3** as dehydrodigallic acid dimethyl ester, a natural product which has not previously been reported in the available current literature.

In addition, the known compounds, isoferulic acid **4** and ferulic acid **5** were also isolated and identified by applying the conventional and spectral methods of analysis.

2.2. Evaluation of the biological activity

During evaluation of the biological activity the aqueous methanol extract and the two major phenyl propanoid constituents, isoferulic acid (**4**) and ferulic acid (**5**) showed a remarkable radical scavenging activity in the DPPH assay. Table 2 demonstrates this effect quantitatively in compari-

Table 2 : Results of the quantitative DPPH assay

Sample (μg/ml)	Radical scavenging activity in %				
	10	50	100	500	1000
Ascorbic acid	32.2	93.8	95.4	95.6	98.3
Methanol extract	13.8	27.0	87.2	92.1	93.3
Ferulic acid	17.7	44.5	84.9	95.1	95.9
Isoferulic acid	2.9	13.2	25.8	95.3	95.4

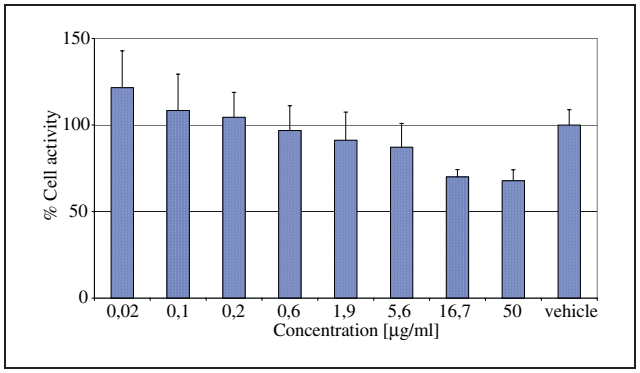


Fig. 1: Cell activity (MTT transformation) of HaCaT cells in response to the aqueous methanol extract of *Tamarix aphylla* flowers or vehicle (medium) measured in MTT assay. Bars represent mean ± SD of two independent experiments

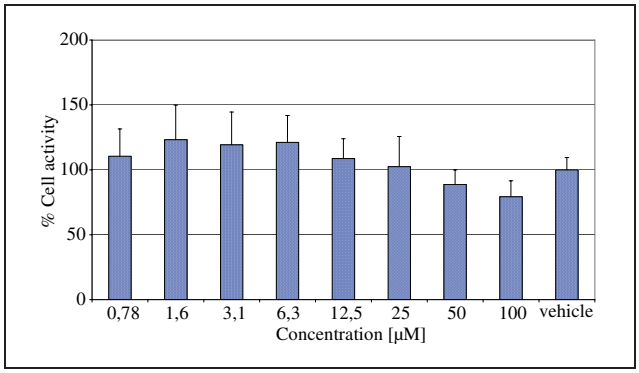


Fig. 3: Cell activity (MTT transformation) of HaCaT cells in response to isoferulic acid or vehicle (medium). Bars represent mean ± SD of three independent experiments

son to those of ascorbic acid. It can be seen that the extract and ferulic acid exhibit a higher radical scavenging capacity than isoferulic acid. Also, cell viability in response to different concentrations of the test substances were determined by MTT-Assay. This assay measures the activity of cellular dehydrogenases to transform the colourless MTT into a blue coloured formazan derivative and indicates the metabolic viability as well as the number of the investigated cells. Treatment with aqueous methanol extract of *Tamarix aphylla* flowers resulted in a reduction of cell viability in a dose dependent manner (Fig. 1). Cytotoxic effects were visible between 0.2 and 0.6 µg/ml and cell viability decreased to 67.8% at 50 µg/ml. However, cell viability at this concentration was well above the IC_{50} limit, so the latter could not be estimated. At lower concentrations a slight cell stimulating effect was observed. Treatment with ferulic acid resulted in a cell stimulating effect in a concentration range between 3.1 and 50 µM (Fig. 2). Concentrations of 3.1 µM and 6.3 µM showed the strongest effect with a stimulation of 142% and 141% respectively of the control. Furthermore, there was no visible reduction in cell viability with increasing doses of ferulic acid. In contrast, doses of 50 µM and 100 µM of isoferulic acid did moderately reduce the cell viability to about 88% and 79%, respectively of the control (Fig. 3), dose dependently. A moderate cell stimulating effect of isoferulic acid was observed in a concentration range between 0.78 µM and 12.5 µM. These results confirm the strong scavenging capacity of ferulic acid which prompted us to investigate the effects of the extract and its main components on cultivated human skin cells in the MTT assay. Whereas higher concentrations of the extract had a weak diminishing effects on the cells. Lower concentrations of extract and isoferulic acid and all concentrations of ferulic acid stimulated the cell activity. This corresponds to the known anti-inflammatory and UV protecting effects (Saija et al. 2000) of phenyl acrylic acids and their esters. Probably not only radical scavenging activity of ferulic and isoferulic acid, but also their affinity to other cellular targets might be important for their activity on keratinocytes.

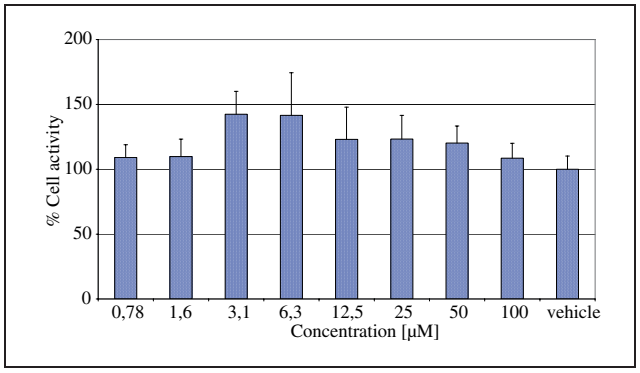


Fig. 2: Cell activity (MTT transformation) of HaCaT cells in response to ferulic acid or vehicle (medium). Bars represent mean ± SD of three independent experiments

3. Experimental

3.1. Instruments and materials

^{1}H NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer 500 MHz NMR spectrometer, at 500 MHz. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d6 and converted to TMS scale by adding 39.5. Typical conditions: spectral width = 8 kHz for 1 H and 30 kHz for 13 C, 64 K data points and a flip angle of 45. FTMS spectra were measured on a Finnigan LTQ-FTMS (Thermo Electron, Bremen, Germany) (Department of Chemistry, Humboldt-Universität zu Berlin). UV recording were made on a Shimadzu UV-Visible-1601 spectrophotometer. $[\alpha]_D^{25}$ were measured on a Kruess polarimeter-8001 (A. Kruess Optronic, Germany). Flame atomic absorption analysis was performed on a Varian Spectra-AA220 instrument, lamp current: 5 ma, fuel: acetylene, oxidant: air, slit width: 0.5 nm. Paper chromatographic analysis was carried out on Whatman No. 1 paper, using solvent systems: (1) H_2O; (2) 6 % HOAc; (3) BAW (n-BuOH–HOAc–H_2O, 4 : 1 : 5, upper layer). Solvent 3 was used for PPC.

3.2. Plant material

Fresh flowers of *Tamarix aphylla* were collected on October, 2006 from El-Alamain at the northern coast of Egypt. Authentication was performed by Dr. M. El-Gebali, former researcher of Botany at the National Research Centre (NRC) of Cairo, Egypt. A voucher specimen was deposited at the herbarium of the NRC.

3.3. Preparation of extract

The fresh *T. aphylla* flowers, dried in the shade in an air draft at room temperature (800 g) were refluxed in a MeOH/H_2O (3 : 1) mixture (three extractions, each for 8 h with 1.25 L). The collected solution was filtered and dried *in vacuo* to yield a brownish orange amorphous powder of the aqueous methanol extract (153 g). The extract (145 g) was then refluxed with acetone (1.5 L, 5 h) and filtered on, thus yielding acetone fraction (dried in vacuum, 20 g) and a residue.

3.4. Isolation and identification of phenolics

3.4.1. From the residue

The residue material (100 g) was applied to a Sephadex LH-20 (25–100 µm, GE Healthcare, Sweden), (500 g) column (100 × 5 cm) and eluted with H_2O followed by H_2O–MeOH mixtures of decreasing polarities. Compound **1** (55 mg) was isolated in pure from the column fraction eluted with 30% MeOH, by repeated column fractionation on Sephadex LH-20, using n-BuOH saturated with H_2O for elution. Compound **2** (71 mg) was obtained from the fraction eluted with 10% MeOH through precipitation from H_2O solution by acetone (thrice), and the subsequent prep. PC of the precipitate, using n-BuOH saturated with H_2O as solvent.

3.4.2. From the acetone fraction

The dried material of the acetone fraction (20 g) was applied to a polyamide 6s (250 g) (Riedel-De-Haen AG, Seelze, Hannover, Germany) column (88 × 6.5 cm) and eluted with H_2O followed by H_2O–MeOH mixtures of decreasing polarities. Compounds **3** (38 mg) and **5** (92 mg) were individually separated pure from the material of the fraction eluted with 50% MeOH by applying polyamide column (30 × 2 cm) fractionation, using EtOAc water saturated for elution. Crystallization (twice) from bidistilled H_2O of the material of the fraction eluted with 20% MeOH afforded a pure colorless crystalline sample (120 mg) of compound **4**.

3.4.3. Isoferulic acid 3-O-β-$^4C_1$-glucopyranoside (**1**)

Faint yellow amorphous powder, $[\alpha]_D^{25}$ −41.4° (c = 0.5, MeOH), R_f -values: 0.33 (H2O), 0.68 (HOAc), 0.75 (BAW). UV max nm in MeOH: 235, 292, 309. HRFIMS: m/z = 355.1112 $[M-H]^-$, ($C_{16}H_{20}O_9$). Normal acid hydrolysis gave glucose and isoferulic acid (Co-PC). Isoferulic acid: R_f-values: 0.39 (HOAc), 0.69 (BAW). UV max nm in MeOH: 241, 292, 322. EI-MS: m/z = 194 $[M]^+$. ^{1}H NMR: δ ppm: 7.45 (d, J = 16 Hz, H-7), 7.08 (m, H-2 and H-6), 6.95 (d, J = 8 Hz, H-5), 6.24 (d, J = 16 Hz, H-8) and 3.8 (s, H-OMe). ^{13}C NMR (Table 1). ^{1}H NMR of **1**: δ ppm: glucose moiety: 4.86 (d, J = 8 Hz, anomeric H-1), 3.50–3.90 (m, sugar protons overlapped with water protons); Isoferulic moiety: 7.59 (broad s, $\Delta\nu_{1/2}$ = 4 Hz, H-2); 7.11 (d, J = 16 Hz, H-7); 7.06 (broad d, J = 8 Hz, $\Delta\nu_{1/2}$ = 4 Hz, H-6); 6.90 (d, J = 8 Hz, H-5); 6.18 (d, J = 16 Hz, H-8). NMR data of **1** (Table 1).

3.4.4. Tamarixetin 3,3′-di-sodium sulphate (**2**)

R_f-values: 0.80 (H_2O), 0.69 (HOAc, 0.36 (BAW); Electrophoretic mobility [buffer, pH 2 (2.5% formic acid + 8% acetic acid), 250 V, 20 mA, room temp. on Whatman paper No. 3MM, 90 min]: 6.0 cm; UV max nm in MeOH (A): 242 (inflection), 266, 341; (A) + NaOAC: 257 (inflection), 271, 360; (A) + NaOAc + H_3BO_3: 270, 342; (A) + $AlCl_3$: 257 (inflection), 276, 305, 365 (inflection), 402; (A) + $AlCl_3$ + HCl: 259 (inflection), 276, 302, 430; (A) + NaOMe: 276, 320, 396. Normal acid hydrolysis (2 N aqueous HCl, 1/4 h, 100 °C) yielded tamarixetin: R_f-values: 0.08 (H_2O), 0.17 (HOAc, 0.83 (BAW); UV max nm in MeOH (A): 238,255, 268, 369 (A) + NaOAC: 253 (inflection), 273, 312, 360 (shoulder); (A) + NaOAc + H_3BO_3: 255, 265 (inflection), 368; (A) + $AlCl_3$: 268, 301 (inflection), 363, 430; (A) + $AlCl_3$ + HCl: 268, 301 (inflection), 362, 426; (A) + NaOMe: 268, 422. EI-MS: m/z = 316 $[M]^+$. ^{1}H NMR: δ ppm: 6.22 (d, J = 2 Hz, H-6); 6.45 (d, J = 2 Hz, H-8); 7.08 (d, J = 8 Hz, H-5′); 7.65 (m, H-2′ and H-6′); 3.81 (s, Me-4′). Controlled acid hydrolysis (0.05 N aqueous HCl, 15 min, 100 °C) yielded intermediates **2a** and **2b**.

2a: R_f-values: 0.46 (H_2O), 0.40 (HOAc), 0.33 (BAW); Electrophoretic mobility: 4.2 cm. UV max nm in MeOH (A): 252 (inflection), 267, 343; (A) + NaOAC: 255 (inflection), 272, 388; (A) + NaOAc + H_3BO_3: 254, 267, 350; (A) + $AlCl_3$: 268, 274, 300, 412; (A) + $AlCl_3$ + HCl: 254, 268, 390; (A) + NaOMe: 269, 320, 389. ^{1}H NMR: δ ppm: 6.20 (d, J = 2 Hz, H-6); 6.40 (d, J = 2 Hz, H-8); 7.10 (d, J = 8 Hz, H-5′); 7.62 (m, H-2′ and H-6′), 3.83 (s, Me-4′). **2b**: R_f-values: 0.40 (H_2O), 0.32 (HOAc), 0.56 (BAW); Electrophoretic mobility: 4.0 cm. UV max nm in MeOH (A): 255 (inflection), 267, 343; (A) + NaOAC: 274, 360; (A) + NaOAc + H_3BO_3: 254,267,358; (A) + $AlCl_3$: 274, 300, 380; (A) + $AlCl_3$ + HCl: 2275, 405; (A) + NaOMe: 276, 310, 395. ^{1}H NMR: δ ppm: 6.18 (d, J = 2 Hz, H-6); 6.40 (d, J = 2 Hz, H-8); 6.92 (d, J = 8 Hz, H-5′); 7.85 (d, J = 2 Hz, H-2′); 7.93 (dd, J = 8 Hz, J = 2 Hz, H-6′), 3.85 (s, Me-4′). −ve ESI-MS of **2**: m/z = 497 $[M-Na]^-$, 417 $[M-SO_3Na]^-$, 395 $[417 + H\text{-}Na]^-$, 315 $[417 + H\text{-}SO_3Na]^-$; +pv ESI-MS of **2**: m/z = 521 $[M + H]^+$, 543 $[M + 23]^+$. ^{1}H NMR of **2**: δ ppm: 6.20 (d, J = 2 Hz, H-6); 6.49 (d, J = 2 Hz, H-8); 6.85 (d, J = 8 Hz, H-5′); 7.89 (d, J = 2 Hz, H-2′); 7.97 (dd, J = 8 Hz, J = 2 Hz, H-6′), 387 (s, Me-4′). ^{13}C NMR (Table 1).

3.4.5. Dehydrodigallic acid dimethyl ester (**3**)

R_f-values: 0.58 (H_2O), 0.70 (HOAc), 0.80 (BAW); HPLC/-ve-ESIMS: conditions for HPLC/ESI-MS analysis: A binary gradient with the following time program was used, where the solvents are (A) H_2O–HOAc (98 : 2) and (B) MeOH–H_2O–HOAC (80 : 18 : 2): 0–5 min, 5% B; 5–50 min, increased to 41% B, held for 15 min; 65–80 min, increased to 100% B, held for 5 min; 85–90 min, decreased to 5% B, held for 10 min. The flow rate was 0.2 ml min ∼ 1 and the injection volume was 10 µl. The columns (100 × 2 mm i.d.) were filled with 5 µm Nucleosil 120 C_{18} and for compounds UV detection at 280 nm a Spectra-Physics detector was used. The HPLC instrument was coupled to a MAT95 sector field mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an ESI II ion source: R_t: 22.99 min; $[M-H]^-$ at m/z = 365. UV max nm in MeOH: 274. EI-MS: m/z = 366 $[M]^+$. Normal acid hydrolysis gave dehydrodigallic acid (Co-PC). Dehydrodigallic acid: R_f-values: 0.54 (H_2O), 0.60 (HOAc), 0.72 (BAW). UV max nm in MeOH: 272; EI-MS: m/z = 338 $[M]^+$. ^{1}H NMR: δ ppm 7.02 (d, J = 2.5 Hz, H-2); 6.5 (d, J = 2.5 Hz, H-6); 6.9 (s, H-6′). ^{13}C NMR (Table 1). ^{1}H NMR of **1**: δ ppm: 7.04 (d, J = 2.5 Hz, H-2); 6.6 (d, J = 2.5 Hz, H-6); 6.98 (s, H-6′); 3.63 and 3.55 both aare singlets (protons of two methyl esters). ^{13}C NMR: (Table 1).

3.5. Biological methods

3.5.1. Determination of radical scavenging activity by DPPH assay

Qualitative DPPH assay was made by TLC. Test substances (5 µl, methanol extract, ferulic acid and isoferulic acid) and the positive control ascorbic acid were spotted on a silica gel plate and detected with 1 mM ethanolic DPPH solution (Sigma, Taufkirchen, Germany). Quantitative determination was carried out according to the method of Brand et al. (1995). Different concentrations of test samples in ethanol (10, 50, 100, 500 and 1000 µg/ml) were prepared and 500 µl of each of them were mixed with 500 µl of a 1 mM ethanolic DPPH solution. The samples were incubated for 30 min at room temperature in the dark and the absorbance at 517 nm was measured (UVmini-1240 UV-VIS-Spectrophotometer, Shimadzu, Duisburg, Germany). Ascorbic acid was used as positive control. Experiments were carried out in triplicate. Percent radical scavenging activity by sample treatment was determined by comparison with an ethanol treated control. The radical scavenging activity was calculated as follows:

$$\text{scavenging effect (\%)} = [(A0 - A1) / A0] \times 100$$

where A0 was the absorbance of the control reaction and A1 was the absorbance in the presence of the sample of the tested substance or extract.

3.5.2. Cell culture and assay conditions

The spontaneously transformed non-tumorigenic human keratinocyte cell line HaCaT (kindly provided by Prof. Fusenig of the German Cancer Research Centre, Heidelberg, Germany) was cultured in growth medium at 37 °C with 5% CO_2 in a humidified atmosphere. Growth medium (RPMI 1640) was supplemented with 8% heat inactivated fetal calf serum (FCS, Sigma, Taufkirchen, Germany) and antibiotics (penicillin 100 units ml^{-1}, streptomycin 100 µg ml^{-1}). Medium was changed every three days. Cells were subcultured using EDTA (0.05% in phosphate buffered saline, PBS) and trypsin/EDTA (0.05%/0.02% in PBS). Cells were detached by gently tapping and suspended in growth medium. An aliquot of the cell suspension obtained was incubated in a hypo-osmotic buffer. Cell nuclei were set free and were counted using a Buerker haemocytometer. For the experiments the growth medium was replaced by RPMI 1640 containing 0.01% bovine serum albumine (BSA, Sigma, Taufkirchen, Germany) and penicillin/streptomycin (BSA medium). Cell culture plastics and medium supplements were obtained from Biochrom AG (Berlin, Germany) except otherwise stated.

3.5.3. Cell viability determination

All assays were conducted between passages 60 and 70. HaCaT cells were plated in 96 well plates in growth medium in a density of 2 × 104 cells per well. After 24 h growth medium was replaced by BSA medium and cells were incubated with different concentrations of *Tamarix aphylla* flower extract, ferulic acid or isoferulic acid for 72 h at 37 °C. After incubation the cells were observed under the microscope for cell integrity and were treated with MTT solution (BSA medium, final concentration 0.5 mg ml^{-1}) for 3 h at 37 °C. Formazan crystals were dissolved in DMSO and optical density (OD) was measured at 550 nm using a multi well plate reader (Anthos HTII, Salzburg, Austria). Cell viability was expressed as a percentage of vehicle control.

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