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Cytotoxic ellagitannins from *Reaumuria vermiculata*

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ABSTRACT

Three ellagitannins and one disulfated flavonol were isolated from the aerial parts of *Reaumuria vermiculata* L. Besides that, 16 known compounds were characterized as well. The structures of all compounds were elucidated on the basis of spectroscopic data including 1D and 2D NMR and ESI HR-FTMS. The in vivo antioxidant activity using the oxygen radical absorbance capacity (ORAC) method, of the extract, its column fractions and two of the isolated ellagitannins was accomplished. In addition, a possible cytotoxicity of the extract and two of the new ellagitannins on HaCaT human keratinocytes and the activity of both compounds against the prostate cancer cell line (PC-3) were also assessed, whereby a potent cytotoxicity with IC_{50} less than 1 μg/ml was determined for both compounds. Besides, the extract exhibited a potential cytotoxic effect against four different solid tumor cell lines, namely liver (Huh-7), colorectal (HCT-116), breast (MCF-7) and prostate (PC-3). The IC_{50}s were found to be substantially low (ranged from 1.3±0.15 to 2.4±0.22 μg/ml) with relatively low resistance possibility reaching to 0% in the case of Huh-7 cell.

1. Introduction

Laboratory investigations show that the biological activities of some plant phenolics, specially ellagitannins and flavonoids are most probably due to their strong antioxidant properties [1,2]. Several plant families are known to be rich in ellagitannins and flavonoids. Among these families, the Tamaricaceae includes species which provide extracts rich in ellagitannins and flavonoids. Egyptian Tamaricaceous plants belong to two definite genera, namely, *Tamarix* and *Reaumuria* [3]. While almost all parts of the existing five *Tamarix* species and of the species *Reaumuria hirtella* have been comprehensively studied for their phenolics [4–6], only preliminary phytochemical investigations [7] have been carried out on *Reaumuria vermiculata*, one of the four *Reaumuria* species existing in Egypt. Many of these plants grow on saline soils, tolerating up to 15,000 ppm soluble salt and can also tolerate alkaline conditions. In view of this fact, the capability of these plants on synthesizing and accumulating sulfate conjugates of flavonols phenyl propanoids and other phenolics [6c), d), g), m)], is thus not all that surprising. These compounds are produced via the sulfotransferase (SOT) enzymes [8] which catalyze the sulfonation of a wide range of compounds, including flavonoids and phenols, and produce the more water-soluble sulfate esters and sulfate conjugates. It is hypothesized that sulfonation, via SOTs, enzymes, affects the biological activity of certain compounds, thereby modulating physiological processes such as growth, development, and adaptation to salinity stress [9–11]. Besides, the Tamaricaceae plants are capable of synthesizing and accumulating a wide variety of phenolics, including lignans [6e)], phenolic glycerides [6i)], gallotannins [6k)], monomeric and oligomeric ellagitannins [5,6l)] and flavonol glucuronoids

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[6g)]. Tamaricaceous plants have been used as folk medicines mainly as anti-inflammatory, antiseptic and anti-pyretic agents [12]. Among these plants, *R. vermiculata* L. (=*Reaumuria mucronata* Jaub. & Spach), known in English as saltwort-leaved *Reaumuria* is an elegant little shrub which grows wild in the Mediterranean coastal strip of Egypt. Its leaves are numerous, scattered, sessile from a quarter to three quarters of an inch long, 4 spreading linear-a whapped, acute, fleshy, smooth, glaucous, convex beneath, flat above, and dotted on both sides with minute depression. The white flowers are terminal and solitary. Calyx leaves are ovate with a narrow entire membranous edge. The petals terminate in three slight lops. Its capsule is brown, very smooth and somewhat shining, and its valves are reflexed after the seeds are discharged [3]. In Egypt, the plant is used as a cure from itch and bruises by being applied externally or taken internally in the form of a decoction [12]. The plant has been reported previously to contain kaempferol 3,7-disulfate, on the basis of chromatographic, electrophorotic, and UV spectral analysis and results of hydrolysis [7]. The present study has been undertaken to investigate in detail the constitutive phenolics of *R. vermiculata* aqueous methanol aerial part extract in association with its antioxidant capability, cytotoxicity and anticancer activity. During the course of this work, we were able to isolate and determine the structure of twenty phenolics including four metabolites which have not been reported previously to occur as natural products, namely, tamarixetin 3,7-disulfate (**2**), 3-methoxyellagic acid 4,4′-disulfate (**4**), 2-*O*-dehydrodigallic acid monocarboxyloyl-3-*O*-galloyl-(α/β)-glucose (**9**) and the ellagitannin dimer, vermaculitin (**13**). On the other hand, the activity of the extract, its column fractions and the two isolated ellagitannins **9** and **13** for the radical scavenging capacity using the DPPH method [13] and the ORAC assay [14] has been evaluated. In addition, the possible cytotoxicity of the extract, and the two ellagitannins **9** and **13** against HaCaT human keratinocytes, the potential cytotoxic effect of the extract against four different solid tumor cell lines and the cytotoxic effect of metabolites **9** and **13** against prostate cancer cell line were also assessed [15].

2. Experimental

2.1. General experimental procedures

^{1}H NMR spectra were measured using a Jeol ECA 500 MHz NMR spectrometer, at 500 MHz. ^{1}H chemical shifts (δ) were measured in ppm, relative TMS and ^{13}C NMR chemical shifts to DMSO-d_6 and were converted to TMS scale by adding 39.5. High resolution ESI mass spectra were measured using a Finnigan LTQ FT Ultra mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanomate ESI interface (Advion). An electrospray voltage of 1.7 kV (+/−) and a transfer capillary temperature of 200 °C were applied. Collision induced dissociation (CID) was performed in the ion trap using a normalized collision energy of 35%, activation time of 30 ms, 0.25 activation Q and a precursor ion isolation width of 2 amu. High resolution product ions were detected in the Fourier transform ion cyclotron resonance (FTICR) cell of the mass spectrometer. UV recording was made on a Shimadzu UV–Visible-1601 spectrophotometer. Flame atomic absorption analysis was performed on a Varian Spectra-AA220 instrument, lamp current: 5 mA, fuel: acetylene, oxidant: air, and slit width: 0.5 nm. Paper chromatographic analysis (PC) 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).

2.2. Plant materials

Collection of the fresh aerial parts of *R. vermiculata* was made at the Mediterranean costal region, 5 km west of Mersa-Matrooh, Egypt, on April, 2010. Authentication was performed by Dr. S. Kawashty, Prof. of Botany at the National Research Center (NRC) of Cairo. Voucher specimen was deposited at the herbarium of the NRC.

2.3. Preparation of extract

The fresh aerial parts of *R. vermiculata*, dried in the shade in an air draft at room temp. (2 kg) were comminuted to powder and homogenized with a MeOH/H_2O (3:1) mixture, filtered, and dried in vacuum to yield a dark brown amorphous powder (290 g).

2.4. Isolation and identification of phenolics

A portion (97.5 g) of the aqueous MeOH extract thus obtained, was applied to a Sephadex LH-20 (850 g) column (100×7.5 cm) and eluted with H_2O followed by H_2O/MeOH mixtures of decreasing polarities, whereby, twelve fractions (I–XII) were individually desorbed, dried in vacuum and subjected to two dimensional paper chromatography (2DPC). Compounds **1** (87 mg) and **2** (54 mg) were isolated pure from 1.36 g of fraction II (eluted from the major column with 10% aq. MeOH), by column fractionation (CF) over 15 g of Sephadex LH-20 using a MeOH/H_2O mixture for elution. Compounds **3** (64 mg) and **4** (92 mg) were separated pure from 698 mg of fraction III (eluted with 20%), by fractionation on a Sephadex LH-20 (13 g) column using 40% aqueous MeOH for elution. Compounds, **5** (102 mg), **6** (92 mg) and **7** (54 mg) were individually separated pure from 601 mg of fraction IV (eluted with 30%) by prep. PC, using *n*-BuOH water saturated as solvent. Compounds **8** (44 mg), **9** (103 mg) and **10** (88.7 mg) were isolated pure from 767 mg of fraction V (eluted with 40%) by applying a polyamide column (35 g) fractionation and gradient elution with water/MeOH mixture of decreasing polarities, whereby, three successive subfractions were individually eluted, subfraction i, by 20%; ii, by 30% and iii, by 50% aq. MeOH, respectively. Purification by prep. PC using BAW (*n*-BuOH–HOAc–H2O, 4:1:5, upper layer) as solvent afforded pure samples of compounds **8** (48 mg) from i, **9** (33 mg) from ii and **10** (17 mg) from iii. 2DPC and CoPC of fraction VI proved the presence of compound **9** as a main constituent, among other minors. This fraction was therefore not further processed. Compound **11** (91 mg) was obtained from 372 mg of fraction VII (eluted with 60%) through repeated (thrice) crystallization from aq. MeOH (50%). Sephadex LH-20 column of 286 mg of fraction VIII (eluted with 70%), using *n*-BuOH saturated with H_2O for elution yielded a pure sample of compound **12** (122 mg). Compound **13** was obtained from 717 mg of fraction IX (eluted with 80%) through an MCI gel (CHP-20P) column fractionation, using gradient elution with H_2O/MeOH mixtures of decreasing polarities. The obtained subfractions were examined by 2DPC

(two dimensional paper chromatography). The major subfraction vii, eluted with 90% was dried under vacuum at 45 °C, dissolved in acetone, and filtered and the filtrate was treated with diethyl ether in excess to yield an off-white precipitate. Repeated precipitation (thrice) afforded a pure sample (189 mg) of **13**. The material, 722 mg of fraction X (eluted with 90%) was fractionated over a polyamide-s6, using a solvent mixture of MeOH:Benzen:H_2O (60:38:2) for elution, and the individually collected crude materials were separately purified by crystallization from EtOH, thus yielding pure samples of compounds, **14** (33 mg), **15** (19 mg) and **16** (46 mg). Compounds **17** (26 mg), and **18** (19 mg) were individually isolated from 167 mg of fraction XI (eluted with MeOH) through repeated fractional crystallization (thrice). The material, 123 mg of fraction XII (also, eluted with MeOH) was subjected to repeated prep. PC, using 30% of aq. acetic acid as solvent, whereby pure samples of compounds **19** (25 mg) and **20** (34 mg) were isolated pure.

*2.4.1. Tamarixetin 3,7-disulfate (**2**)*

A light yellow amorphous powder, R_f-values: 0.85 (H_2O), 0.73 (HOAc), 0.20 (BAW). Electrophoretic mobility: 5.6 cm, on Whatman No. 3 MM paper, buffer solution of pH 2, H_2O–HCOOH–AcOH (89:8.5:2.5), 1 and 1/2 h, 50 V/cm. UV λ_{max} nm in MeOH: 252, 267, 290, 349; NaOMe: 250, 265 (shoulder), 342; NaOAc: 250, 268, 350; NaOAc–H_3BO_3: 255, 268, 350; $AlCl_3$: 272, 300, 350 (shoulder), 396; $AlCl_3$ + HCl (30 min): 253, 267, 368. Complete acid hydrolysis of **2** (14 mg in 5 ml, 0.1 N aq. HCl, at 100 °C for 15 min) yielded Na_2SO_4 ($BaCl_2$ test and atomic absorption analysis) and quercetin 4′-methyl ether, tamarixetin (5 mg), filtered from the cooled hydrolysate: R_f-values: 0.08 (H_2O), 0.17 (HOAc), 0.83 (BAW); UV λ_{max} nm in MeOH: 238, 255, 268, 369; NaOAC: 253 (inflection), 273, 312, 360 (shoulder); NaOAc–H_3BO_3: 255, 265 (inflection), 368; $AlCl_3$: 268, 301 (inflection), 363, 430; $AlCl_3$ + HCl: 268, 301 (inflection), 362, 426; NaOMe: 268, 422. ^{1}H NMR: δ ppm: 6.22 (1H, *d*, *J* = 2 Hz, H-6); 6.45 (1H, *d*, *J* = 2 Hz, H-8); 7.08 (1H, *d*, *J* = 8 Hz, H-5′); 7.65 (m, H-2′ and H-6′); 3.81 (*s*, Me-4′). Controlled acid hydrolysis (18 mg, 10% aq. AcOH, 15 min, 100 °C) yielded intermediates **2a** (6 mg) and **2b** (4 mg). **2a**: R_f-values: 0.46 (H_2O), 0.40 (HOAc), 0.33 (BAW); electrophoretic mobility: 2.5 cm. UV λ_{max} nm in MeOH 250, 265, 363 (shoulder); NaOAC: 252, 264, 364; NaOAc + H_3BO_3: 254, 267, 358; $AlCl_3$: 263, 300, 345, 420; $AlCl_3$ + HCl: 265, 365, 405; NaOMe: 253, 356, 410. ^{1}H NMR: δ ppm: 6.55 (1H, *d*, *J* = 2 Hz, H-6); 6.94 (1H, *d*, *J* = 2 Hz, H-8); 6.88 (1H, *d*, *J* = 8 Hz, H-5′); 7.92 (1H, *d*, *J* = 2 Hz, H-2′); 7.65 (1H, *dd*, *J* = 8 Hz, *J* = 2 Hz, H-6′), 3.85 (s, Me-4′). **2b**: R_f-values: 0.54 (H_2O), 0.45 (HOAc), 0.56 (BAW); electrophoretic mobility: 2.7 cm. UV λ_{max} nm in MeOH: 252 (inflection), 267, 343; NaOAC: 255 (inflection), 272, 388; NaOAc + H_3BO_3: 254, 267, 345; $AlCl_3$: 268, 274, 300, 412; $AlCl_3$ + HCl: 254, 268, 390; NaOMe: 269, 320, 389. ^{1}H NMR: δ ppm: 6.20 (1H, *d*, *J* = 2 Hz, H-6); 6.40 (1H, *d*, *J* = 2 Hz, H-8); 7.10 (1H, *d*, *J* = 8 Hz, H-5′); 7.62 (m, H-2′ and H-6′), 3.83 (s, Me-4′). ESI-FTMS (negative ions) of **2**: $m/z = m/2 = 236.97871 = [M-2Na]^{2-}$, calc: 236.97869 corresponding to a molecular formula of $C_{16}H_{10}O_{13}S_2$. ^{1}H NMR of **2**: δ ppm: 6.59 (1H, *d*, *J* = 2 Hz, H-6); 6.98 (1H, *d*, *J* = 2 Hz, H-8); 6.90 (1H, *d*, *J* = 8 Hz, H-5′); 7.94 (1H, *d*, *J* = 2 Hz, H-2′); 7.66 (1H, *dd*, *J* = 8 Hz, *J* = 2 Hz, H-6′), 3.84 (s, Me-4′). ^{13}C NMR of **2**: δ ppm: 158.72 (C-2), 132.34 (C-3), 179.69 (C-4), 161.00 (C-5), 103.05 (C-6), 158.72 (C-7), 98.59 (C-8), 155.81 (C-9), 106.98 (C-10), 122.56 (C-1′), 114.80 (C-2′), 146.00 (C-3′), 149.10 (C-4′), 111.50 (C-5′), 126.40 (C-6′), 56.1 (C-OMe).

*2.4.2. 3-Methoxy ellagic acid 4,4′-di-sulfate (**4**)*

R_f-values: 0.72 (H_2O), 0.60 (HOAc), 0.33 (BAW). Electrophoretic mobility: 5.0 cm UV λ_{max} nm in MeOH: 248, 335, 349. Complete acid hydrolysis (14 mg in 5 ml, 0.1 N aq. HCl, at 100 °C for 15 min) yielded Na_2SO_4 ($BaCl_2$ test and atomic absorption analysis) and ellagic acid 3-methyl ether **4a**, filtered on from the cooled hydrolysate: R_f-values: 0.00 (H_2O), 0.07 (HOAc), 0.71 (BAW); UV λ_{max} nm in MeOH: 250, 346, 363. ^{1}H NMR of **4a**: δ ppm: 7.47 (1H, *s*, H-5), 7.42 (1H, *s*, H-5′), 4.00 (3H, *s*, OMe-3). ^{13}C NMR of **4a**: δ ppm: 112.36 (C-1), 141.98 (C-2), 140.64 (C-3), 148.63 (C-4), 111.84 (C-5), 112.66 (C-6), 159.40 (C-7), 112.98 (C-1′), 136.63 (C-2′), 140.21 (C-3′), 152.63 (C-4′), 110.83 (C-5′), 107.85 (C-6′), 159.30 (C-7′), 61.45 (OMe-3). ESI-FTMS (negative ions) of the parent compound **4**: HR ICR MS: $m/z = m/2 = 236.9605 = [M-2Na]^{2-}$, corresponding to the molecular formula $C_{15}H_6O_{14}S_2$ (calc. 236.9601). Controlled acid hydrolysis of **4** (22 mg, aq. 10% AcOH, 100 °C, 10 min) yielded **4b**: R_f-values: 0.42 (H_2O), 0.36 (HOAc), 0.39 (BAW). Electrophoretic mobility: 2.0 cm, UV λ_{max} nm in MeOH: 250, 345, 369; ^{1}H NMR: δ ppm: 8.04 (1 H, *s*, H-5), 7.47 (1H, *s*, H-5′), 4.1 (3H, *s*, OMe-3); ^{1}H NMR and HMBC of **4**: δ ppm: 8.15 (1H, *s*, H-5, HMBC: cross peaks with C-1, C-3, C-4 and C-6); 8.05 (1H, *s*, H-5′, HMBC: cross peaks with C-1′, C-3′, C-4′ and C-6′); 4.10 (3H, *s*, OMe-3, HMBC: cross peak with C-3). ^{13}C NMR of **4**: δ ppm: 117.3 (C-1), 141.9 (C-2), 144.4 (C-3), 146.1 (C-4), 118.6 (C-5), 109.4 (C-6), 161.0 (C-7), 115.8 (C-1′), 139.0 (C-2′), 157.6 (C-3′), 146 (C-4′), 118.2 (C-5′), 113.0 (C-6′), 161.8 (C-7′), 61.95 (C-OMe-3).

*2.4.3. 2-O-dehydrodigallic acid monocarboxyloyl-3-O-galloyl-(α/β)-glucose (**9**)*

R_f-values: 0.39 (H_2O), 0.32 (HOAc), 0.14 (BAW); UV λ_{max} nm in MeOH: 272; negative ESIMS, $[M-H]^- = 633$, corresponding to *m/r* 634 and molecular formula of $C_{27}H_{22}O_{18}$. Complete acid hydrolysis (52 mg in 10 ml aq. 1.5 N HCl, 100 °C, 5 h) of **9** yielded glucose (CoPC), and gallic and dehydrodigallic acids; gallic acid: R_f-values: 0.53 (H_2O), 0.53 (HOAc), 0.78 (BAW); UV λ_{max} nm in MeOH: 272; ^{1}H NMR: δ ppm: 6.96 (1H, *s*, H-2 and H-6); ^{13}C NMR: δ ppm: 120.6 (C-1), 108.8 (C-2 and C-6), 145.5 (C-3 and C-5), 138.1 (C-4); dehydrodigallic acid: R_f-values: 0.55 (H_2O), 0.60 (HOAc), 0.78 (BAW); UV λ_{max} nm in MeOH: 272; ^{1}H NMR: δ ppm: 7.03 (1H, *d*, *J* = 2.5 Hz, H-2), 6.5 (1H, *d*, *J* = 2.5 Hz, H-6), 6.9 (1H, *s*, H-6′); ^{13}C NMR: δ ppm: 120.6 (C-1), 111.3 (C-2), 148 (C-3), 139.6 (C-4), 146.1 (C-5), 107.1 (C-6), 168.2 (C-7), 115.7 (C-1′), 136.6 (C-2′), 140.0 (C-3′), 139.7 (C-4′), 143.0 (C-5′), 109.0 (C-6′), 167.1 (C-7′). Controlled acid hydrolysis (25 mg of **9**, aq. 1 N HCl, 100 °C, 3 h) gave intermediate **9a**: R_f-values: 0.55 (H_2O), 0.72 (AcOH), 0.33 (BAW); negative ESIMS: $m/z = 331$, $[M-H]^-$, $Mr = 332$; λ_{max} in MeOH at 273 nm. ^{1}H NMR of **9**: α-glucose moiety: δ ppm: 5.63 (*t*, *J* = 9 Hz, H-3-α), 5.25 (*d*, *J* = 3.5 Hz, H-l-α), 4.82 (*dd*, *J* = 9 Hz and 3.5 Hz, H-2-α), 3.6–3.95 (m, H-4, H-5 and H-6 in this moiety); β-glucose moiety: δ ppm: 5.2 (*t*, *J* = 8 Hz, H-3-β), 4.96 (*t*, *J* = 8 Hz, H-2-β), 4.38 (*d*, *J* = 8 Hz, H-1-β) 3.95 (*m*, H-5-β), 3.6–95 (*m*, H-4 and 2H-6); galloyl moieties: 7.04, 6.99 (each *s*, H-2 and H-6 in both moieties); dehydrodigalloyl moieties: δ ppm: 7.14, 7.13 (each *d*, *J* = 2.5 Hz, H-2 in both moieties), 6.66, 6.64 (each *d*, *J* = 2.5 Hz, H-6 in both moieties), 6.96, 6.94 (each *s*, H-6′ in both moieties); COSY results (see Results and discussion part).

^{13}C NMR of **9**: δ ppm: α-glucose moiety: 89.79 (C-l), 72.07 (C-2), 71.69 (C-3), 68.53 (C-4), 73.07 (C-5), 60.88 (C-6); β-glucose moiety: 94.58 (C-l), 73.25 (C-2), 77.08 (C-3), 68.35 (C-4), 76.33 (C-5), 60.88 (C-6); galloyl moieties: 123.40, 122.80 (C-l), 109.55, 109.45 (C-2 and C-6), 145.08, 145.03 (C-3 and C-5), 138.61, 138.39 (C-4), 166.86, 167.45, (C═O); dehydrodigalloyl moieties: 120.13, 119.88 (C-1), 111.42, 1141 (C-2), 146.99, 146.76 (C-3), 139.96 (C-4), 145.24, 145.03 (C-5), 106.97, 106.88 (C-6), 164.42, 163.82 (C═O, 7), 113.41, 113.12 (C-1′), 136.48, 137.13 (C-2′), 1140.07, 139.82, (C-3′), 140.07 (C-4′), 142.48, 142.49 (C-5′), 109.00, 109.01 (C-6′), 166.88, 167.37 (C═O, 7′).

2.4.4. Vermiculatin (**13**)

An off-white amorphous powder, R_f-values: 0.42 (H_2O), 0.38 (HOAc), 0.16 (BAW). $[\alpha]_D^{25}+34$ (MeOH, $c=1$). UV λ_{max} MeOH nm: 224, 278. Complete acid hydrolysis of **13** (62 mg, 2 N aq. HCl, 100 °C, 5 h) yielded glucose (CoPC), and gallic and dehydrodigallic acids (CoPC, ^{1}H and ^{13}C NMR). Controlled aqueous hydrolysis of vermiculatin (65 mg, 20 ml H_2O, 100 °C, 12 h) yielded **13a**, (2DPC), separated pure (17 mg) by MCI-gel (CHP-20P) column fractionation of the dried aq. hydrolysate and elution with H_2O/MeOH mixtures of decreasing polarities. **13a** was identified to be 2-O-dehydrodigalloyl-3-O-galloyl-(α/β)-glucose (CoPC against compound **9**, ^{1}H and ^{13}C NMR). FTESI-MS (positive ions) of the parent compound, **13**: $m/z=$ 1139 $[M+Na]^+$, HR-ICRMS: $m/z=1139.1409$ corresponding to the molecular formula $C_{47}H_{36}O_{31}Na$; calculated: 1139, 1395. ^{1}H and ^{13}C NMR data of **13**: Table 1.

2.5. Biological methods

2.5.1. Determination of radical scavenging activity by DPPH assay

Radical scavenging activity of plant extract against the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically by the DPPH assay. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep-violet to light-yellow) were measured at 517 nm. The determination was done by a slightly modified method of Brand-Williams et al. [13], where the extract solution was prepared by dissolving 0.025 g of the dry extract in 10 ml of methanol. The solution of DPPH in methanol (6×10^{-5}M) was freshly prepared, before UV measurements. 3 ml of this solution was mixed with 9 different concentrations of the samples. The resulting solutions were kept in the dark for 30 min at room temperature and then the decrease in absorbance was measured.

Absorbance of the blank sample containing the same amount of methanol and DPPH solution was prepared and measured. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula: % inhibition $=[(AB-AA)/AB]\times100$, where: *AB* is the absorbance of the blank sample and *AA* is the absorbance of the tested samples. IC_{50} values, the concentration of the substrate that causes 50% loss of the DPPH activity (color), were calculated for the standard and the extract from a graph plotted for the % inhibition against the concentration in μg/ml.

2.5.2. Determination of oxygen radical absorbance capacity by ORAC assay

ROS are generated by the thermal decomposition of [2,2″-azobis(2-amidinopropane) dihydrochloride (AAPH)] and over time quench the signal of the fluorescent probe fluorescein. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of the fluorochrome. Briefly, 6- hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) which was used as the positive control and test compounds **13** and **9** were dissolved/diluted in a phosphate buffered saline (10 mM, pH 7.4). In each well of a 96 well plate 150 μl of 10 nM fluorescein, 25 μl of Trolox (0.2–3.13 μM) or 25 μl of the test compound was pipetted in quadruplicate. The plate was allowed to equilibrate at 37 °C for 30 min. After incubation, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 90 s to determine the background signal. After 3 cycles, 25 μl (240 mM) of AAPH was injected in each well. Measurements were continued for 90 min. The half life time of fluorescein was determined using MS Excel software.

2.5.3. Viability/cytotoxicity, MTT assay

The spontaneously transformed non-tumorigenic human keratinocyte cell line HaCaT (kindly provided by Prof. Fusenig of the German Cancer Research Centre, Heidelberg, Germany) was used for determination of the viability and metabolic activity of non-tumorigenic cells under the influence of the test samples. Cell culture plastics and medium supplements were obtained from Biochrom AG (Berlin, D), except if otherwise stated. Cells were cultured in a 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, D) and antibiotics (penicillin 100 units ml, streptomycin 100 μg/ml). The medium was changed every 3 days. Cells were subcultured routinely using EDTA (0.05% in phosphate buffered saline, PBS) and trypsin/EDTA (0.05%/0.02% in PBS). For the experiments, the growth medium was replaced by RPMI 1640 containing 0.01% bovine serum albumine (BSA, Sigma Taufkirchen, D, BSA medium) and penicillin/streptomycin (BSA medium). HaCat cells between passages 50 and 70 were plated in 96 well plates in growth medium in a density of 2×104 cells per well. After 24 h the medium was replaced by the BSA medium and the cells were incubated with different concentrations of extract and compounds **13** and **9** for 72 h. After incubation the cells were observed under the microscope for cell integrity and were treated with MTT solution (in BSA medium, final concentration of 0.5 μg/ml). Formazan crystals were dissolved with DMSO and the optical density was measured with a multi well plate reader (BMG Omega, BMG Labtech, Offenburg, D) at 550 nm. Cell viability was expressed as the percentage of vehicle control. Data were analyzed by Mann–Whitney test using SPSS13. A $p<0.05$ value was considered significant. Results were given as mean + SD for 4 independent experiments with 6 replicates for each measurement. As a positive control, 100 μg/ml of *Aloe vera* extract [unformulated, freeze-dried *A. vera* inner gel powder (AV, supplied by Aloe Vera of America Inc., USA or Rainbow Naturprodukte GmbH, D)] was used.

2.5.4. Culture of solid tumor cells

Human hepatocellular carcinoma cell line, Huh-7, colorectal adenocarcinoma cell line (HCT-116), breast adenocarcinoma cell

Table 1
NMR spectral data of vermiculatin (**13**) in DMSO-d_6 (500 MHz for ^{1}H and 125.7 MHz for ^{13}C).

	δ_H (*J*, Hz)	δ_C (ppm) [a,b]	Long range ^{1}H–^{13}C correlations[c]
Glucose I			
1	4.80 (*d*, *J*=8.5 Hz)	92.8 *d*	H-1 to C-2, C-3, C-5
2	4.75 (*dd*, *J*=8.5 and 9.5 Hz)	71.8 *d*	
3	3.72 (*t*, *J*=9.5 Hz)	74.0 *d*	
4	3.35 (*t*, *J*=9.5 Hz)	69.8 *d*	
5	3.6 (*m*)	77.3 *d*	H-4 to C-1, C-3
6	3.68 (*dd*, *J*=4 and 12 Hz) 3.82 (*dd*, *J*=2 and 12 Hz)	60.77 *t*	
Glucose II			
1‴	4.96 (*d*, *J*=8.5 Hz)	92. 6 *d*	H-1‴ to C-2‴, C-3‴, C-5‴
2‴	5.01 (*dd*, *J*=8.5 and 9.5 Hz)	69.2 *d*	
3‴	5.29 (*t*, *J*=9.5 Hz)	75.7 *d*	
4‴	3.68 (*t*, *J*=9.5 Hz). 69.4 *d*		
5‴	3.46 (*m*)	77.4 *d*	H-5‴ to C-1‴, C-3‴
6‴	3.78 (*dd*, *J*=4 and 12 Hz) 3.70 (*dd*, *J*=2 and 12 Hz)	60.77 *t*	
Dehydrodigallic-dicarboxyloyl moieties			
1″ and 1‴		118.63, 117.65 all *s*	
2″ and 2‴	7.02 and 7.05 (both *d* and *d*, *J*=2.5 Hz, H-2″, H-2‴)	111.40, 111.45 both *d*	H-2″ to C-4″, C-6″, C-7″; H-2‴ to C-4‴, C-6‴, C-7‴′
3″ and 3‴		147.55, 147.61 all *s*	′
4″ and 4‴		139.04 *s*	
5″ and 5‴		145.17, 145.18 all *s*	
6″ and 6‴	6.02 and 5.99 (both *d* and *d* *J*=2.5 Hz, H-2″, H-2‴)	104.96, 104.99 all *s* Both *d*	H-6″ to C-2″, C-4″, C-7″; H-6‴ to C-‴2 and C-4‴ C-7″
8″ and 8‴		113.10, 112.98 all *s*	
9″ and 9‴		135.24, 135.09 all *s*	
10″ and 10‴		138.99, 139.04 all *s*	
11″ and 11″		139.05 *s*	
12″ and 12‴		142.32 *s*	
13″ and 13″	7.22 and 7.16 (both *s*)	109.33 *d*	H-13″ to C-9″, C-11″, C-14″; H-13‴ to C-9‴, C-11‴, C-14‴
C═O in both moieties			
7″ and 7‴ and 14″ and 14‴		164.20, 163.91, 163.80, 163.73 all *s*	
Galloyl moiety			
1⁗		119.62 *s*	
2‴ and 6‴		109.46 *d*	C-4‴, C-6‴′, C-7‴
3‴ and 5‴		145.10 *s*	
4‴		138.68 *s*	
7‴		166.37	

[a] Assignments by HMBC and HMQC.
[b] Multiplicities by DEPT.
[c] H–C.

line, MCF-7, and prostate adenocarcinoma cell line, PC-3, were obtained from Vaccera (Giza, Egypt). Cells were maintained in RPMI-1640 supplemented with 100 μg/ml of streptomycin, 100 units/ml of penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere at 37 °C.

2.5.5. Cytotoxicity assays against tumor cells

The cytotoxicity of the *Reaumuria* extract and two of the new compounds, **9** and **13** was tested against tumor cells by SRB assay as previously described [15]. Exponentially growing cells were collected using 0.25% trypsin–EDTA and plated in 96-well plates at 1000–2000 cells/well. Cells were exposed to the crude extract or purified compound for 72 h and subsequently fixed with TCA (10%) for 1 h at 4 °C. After several washing, cells were exposed to 0.4% SRB solution for 10 min in a dark place and subsequently washed with 1% glacial acetic acid. After drying overnight, Tris–HCl was used to dissolve the SRB-stained cells and the color intensity was measured at 540 nm.

2.5.5.1. Data analysis. The dose response curve of the compounds was analyzed using the E_{max} model (Eq. (1)).

$$\%\ \text{cell viablility} = (100-R) \times \left(1-\frac{[D]^m}{K_d^m + [D]^m}\right) + R \qquad (1)$$

where [R] is the residual unaffected fraction (the resistance fraction), [D] is the drug concentration used, [K_d] is the drug concentration that produces a 50% reduction of the maximum inhibition rate and [m] is a Hill-type coefficient. IC_{50} was defined as the drug concentration required to reduce fluorescence to

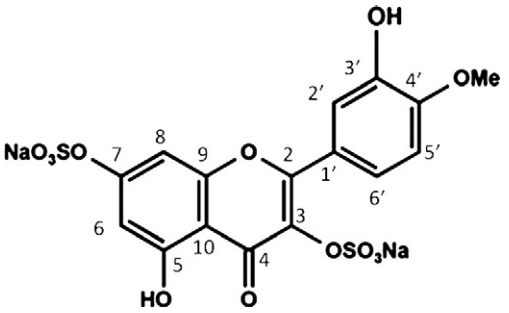
50% of that of the control (i.e., $K_d = IC_{50}$ when R = 0 and $E_{max} = 100 - R$).

2.5.5.2. Statistical analysis. Data are presented as mean ± SD.

3. Results and discussion

Following column chromatographic fractionation of the aqueous methanol extract obtained by homogenizing the aerial parts of *R. vermiculata* in aqueous methanol (75%), 20 compounds (**1**–**20**) were isolated. Conventional and spectral analyses mainly by NMR spectroscopy and by mass spectrometry indicated that four of these compounds are new natural products (**2**, **4**, **9** and **13**).

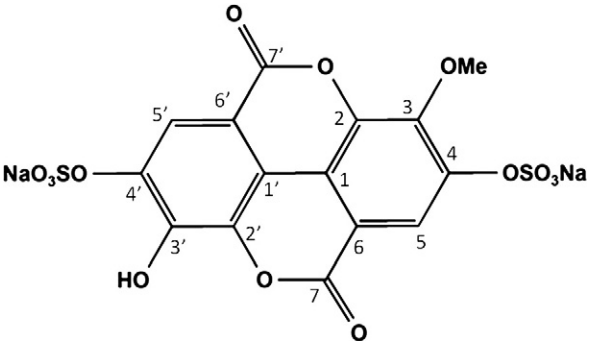
Compound **2**, was isolated as an off-white amorphous powder which exhibited a chromatographic (dark purple spot on PC turning dull yellow when fumed with ammonia, dull yellow when sprayed with Natur-Stuff) and anionic character on electrophoretic analysis similar to those of anionic flavonols [6m), n),16]. UV absorption maxima in MeOH and after addition of diagnostic shift reagents [17,18] showed no shift with NaOAc or with NaOAc/H_3BO_3, a small shift with NaOMe and a 28 nm shift with HCl. These data were consistent with 3,7,4′-trisubstituted quercetin structure. Complete hydrolysis of **2** (0.1 N aq. HCl at 100 °C for 15 min) yielded quercetin 4′-methyl ether, tamarixetin (CoPC, UV, ^{1}H and ^{13}C NMR). The aqueous acidic hydrolysate gave a white ppt. with aq. $BaCl_2$ to prove the presence of the SO_4 group. Atomic absorption analysis confirmed that the SO_4 radical(s) exists in the molecule of **2** as sodium sulfate (S). On controlled acid hydrolysis (10% aq. AcOH, 15 min, 100 °C) **2** yielded two intermediates, **2a** (major, dull yellow spot on PC under UV light) and **2b** (minor, dark purple spot on PC under UV light). Intermediates **2a** and **2b** were individually separated by preparative paper chromatography (prep. PC). Their chromatographic and electrophoretic properties, UV absorption and ^{1}H NMR data proved a 7,4′-disubstituted quercetin structure for **2a** and a quercetin 3,4′-disubstituted structure for **2b**, a result which, when incorporated with the above given analytical data proved that **2** is tamarixetin 3,7-disodium sulfate. Further support for this view was obtained through ^{1}H NMR spectral analysis. The spectrum revealed in the aromatic region a pattern of signals though similar to that of the aglycone, tamarixetin (see Experimental), yet a distinction could be made through the recognition of the downfield shift of the proton signals of H-6 and H-8 (δ ppm 6.60 and 6.99, respectively), in comparison with the signals at δ ppm 6.22 and 6.45 of the corresponding protons in the spectrum of the free aglycone due to substitution at C-7 of the aglycone. Substitution at the C-3 of the aglycone was quite obvious from the dark purple coloration of the spot of **2** on PC. Further confirmation of the structure was received through the mass spectra of **2**. The spectra (negative ions) exhibited a doubly charged *Mr* ion at $m/z = m/2 = 237.33 = [M-2Na]^{-2}$ at low resolution in the ion trap corresponding to $Mr = [(237.33 \times 2) + 2Na] = 520.66$. MSMS analysis of the 237.33 ion showed a doubly charged ion at $m/z = m/2 = 197.1$ indicating a loss of neutral SO_3 from the doubly charged ion. High resolution data obtained in the FTICR cell confirmed the formula $C_{16}H_{10}O_{13}S_2$ from the negative doubly charged ion at $m/z = 236.9787 = [M-2Na]^{-2}$ and the loss of SO_3 thus confirming the structure of compound (**2**) as tamarixetin 3,7-di-sodium sulfate. The achieved structure of **2** was finally confirmed by ^{13}C NMR spectroscopy. From the received spectra, the presence of sulfate substituents attached to positions 3 and 7 was concluded from the upfield shifts of these carbon signals and from the accompanying downfield shift of the corresponding *ortho* and *para*-carbon signals as well, all in comparison with the corresponding chemical shift in the spectrum of the aglycone (see Experimental). Similar shifts are well-known from the work of Markham et al. [19]. The structure of **2** is therefore finally confirmed as tamarixetin 3,7-disodium sulfate, a flavonoid which represents to the best of our knowledge a new natural product. It should be noted however, that this compound was actually obtained previously by semi-synthetic method [20]. The synthetic product showed ^{13}C NMR chemical shifts in DMSO-d_6 similar to those recorded for the natural product.



Compound **2**: Tamarixetin 3,7-di-sodium sulfate.

Compound **4**, an amorphous faint yellow powder which showed chromatographic properties (rosy buff spot on PC under UV light turning yellow on fuming with ammonia) and UV spectral maxima, in MeOH (λ_{max} in MeOH: 248, 335, 349) closely similar to those of ellagic acid derivatives [6h), m)]. **4** exhibited an-ionic characters and yielded on complete acid hydrolysis (aq. 0.1 N HCl, 100 °C, 15 min) compound **4a**, which precipitated pure from the cold hydrolysate and was identified to be ellagic acid 3-monomethyl ether through CoPC, UV, ^{1}H and ^{13}C NMR analysis [20]. The hydrolysate proved by chromatographic analysis, to be free of any sugar, but gave a white precipitate when treated with aq. $BaCl_2$ and proved to contain sodium ions by flame atomic absorption, thus confirming the presence of sodium sulfate(s) in the molecule of **4**. On using ESI MS and detection of negative ions, compound **4** exhibited a doubly charged molecular ion at $m/z = 237.3$ $[M-2Na]^{2-}$, corresponding to an *Mr* of 520 Da. The MSMS analysis of that ion showed loss of a neutral SO_3 unit indicating a sulfate moiety; the structural formulas were established on the basis of high resolution FTICR MS which confirmed a molecular structure of a di-sulfated ellagic acid monomethylether. **4** gave on controlled acid hydrolysis (10% aq AcOH, 100 °C, 10 min), an intermediate **4b**, which was separated

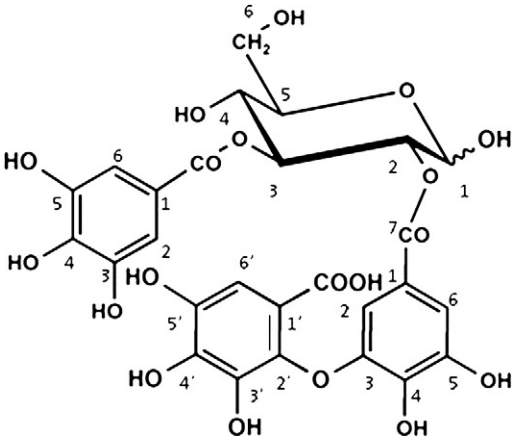
pure by prep. PC, using H_2O as solvent. Chromatographic properties, UV absorption maxima and electrophoretic mobility of **4b** (see Experimental) suggested it to be an ellagic acid 3-methyl ether substituted at either the 4-OH or the 4′-OH by sodium sulfate. This view was supported by ^{1}H NMR analysis of **4b**. The received spectrum showed a pattern of resonances (δ ppm: 8.04 (1H, *s*, H-5), 7.47 (1H, *s*, H-5′), 4.1 (3H, *s*, OMe-3)) which proved that the substituent at either OH-4 or OH-4′ has been released so that the resonance of the vicinal proton, H-5 or H-5′, was shifted upfield to a δ ppm of 7.47. Comparison of the 1D NMR data proved that **4** contained two sulfate substituents, one at OH-4 and the second at OH-4′. This followed from the locations of the resonances of H-5 and H-5′ at δ ppm 8.15 and 8.06, which are recognizably lowfield in comparison with those of the corresponding resonances in the spectrum of the free ellagic acid 3-methyl ether [21]. The ^{13}C NMR data of **4** confirmed its achieved structure. Substitution with sulfates at C-4 and C-4′ is recognized from the upfield resonances of these carbons (δ 146.12 and 146.01 ppm), in comparison with those of the corresponding resonances in the spectrum of the parent compound **4a**. The accompanying downfield shift of the resonances of the β-carbons C-3, C-5, C-3′, and C-5′ are in consistency with this view (see Experimental). Unambiguous assignments of the remaining carbon resonances were based on the results of the HMQC and HMBC spectral analyses. In the HMBC spectrum, ^3J correlations of proton H-5 at 8.15 to C-1 (117.3), C-3 (144.4), and C-7 (161.0) and of H-5′ at δ 8.06 to carbons C-1′ (115.8), C-3′ (157.6), and C-7′ (161.8) were recognized. Correlations of the methoxyl protons (δ 4.1) to the aromatic C-3 (δ 144.42) and of the same downfield aromatic proton (δ 8.15) to the same C-3 carbon confirmed that the site of attachment of one of the sulfate substituent is at the C-4 position of the 3-methoxyellagic acid moiety. The recorded ^3J correlations of H-5′ (δ 8.06) allowed positioning of the second sulfate substituent at C-4′. The recognizable ^2J correlation of this proton to the same C-4′ carbon was in accordance with this conclusion. The complete structure of compound **4** was therefore, determined to be ellagic acid 3-methyl ether 4.4′-di-sodium sulfate.



Compound **4**: ellagic acid 3-monomethyl ether 4,4′-di-sodium sulfate.

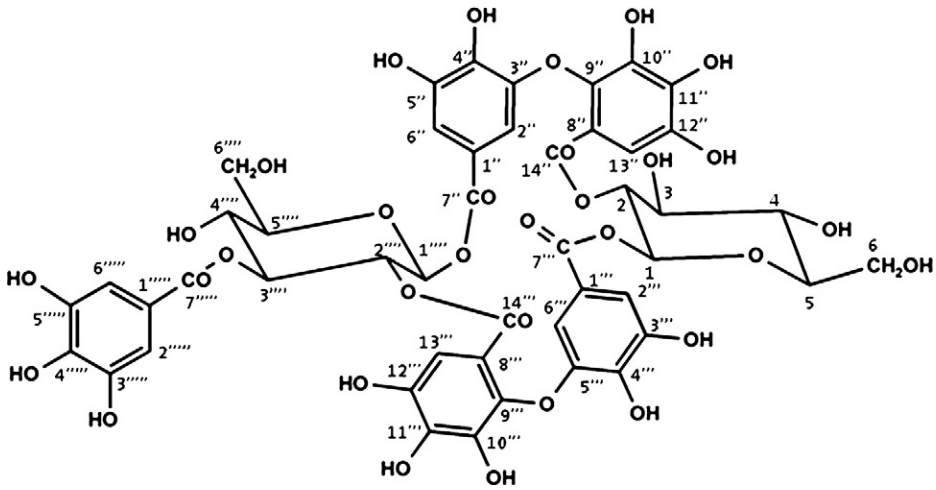
Compound **9**, a white amorphous powder, was found to possess chromatographic properties, color reactions (dark blue spot on PC under short UV, intense blue with $FeCl_3$, pink with KIO_3 [22]) and UV spectra data (λ_{max} in MeOH: 273 nm) consistent with gallotannins. It exhibited an *Mr* of 634, corresponding to a molecular ion $[M-H]^-$ at *m/z* 633. Complete acid hydrolysis (aq. 1.5 N HCl, 100 °C, 5 h) yielded glucose (CoPC), dehydrodigallic acid and gallic acid. MCI-gel (CHP-20P) column fractionation of the dried EtOAc extract of the hydrolysate and elution with H_2O/MeOH mixtures of decreasing polarities yielded individual samples of gallic and dehydrodigallic acids. The identity of both acids was achieved through CoPC, UV, ^{1}H and ^{13}C NMR data [6e)]. On controlled acid hydrolysis (1 N aq. HCl, 100 °C, 3 h) **9** gave, besides glucose, and gallic and dehyrodigallic acids, an intermediate **9a** with chromatographic properties similar to those reported for galloyl esters. **9a** was separated from an EtOAc extract of the hydrolysate by prep. PC and was shown to have an *Mr* of 332 (negative ESIMS, $[M-H]^-$: *m/z* = 331) and a λ_{max} in MeOH at 273 nm. These data show **9a** to be a monogalloyl glucose. The ^{1}H NMR spectrum of **9** revealed two distinct patterns of proton resonances belonging to substituted α and β-glucose anomers. Each pattern was found to contain well separated resonances of the seven-spin system belonging to a distinct glucose anomer, thus proving absence of substitution at the glucose anomeric hydroxyl group. The presence, in this spectrum, of the resonance of the diagnostic glucose proton, H-2-α, downfield at 4.82 (*dd*, *J* = 9 Hz and 3.5 Hz, H-2-α) proved acylation at this hydroxyl group. Measurement of the COSY spectrum for **9** confirmed this finding and proved additional acylation at the 3-hydroxyl of the glucose moiety, whose proton was found to be resonating downfield at δ 5.36 (*t*, *J* = 9 Hz, H-3-α), 5.2 (*t*, *J* = 8 Hz, H-3-β), while the resonance of the H-2-β proton was found to resonate at δ 4.96 (*t*, *J* = 8 Hz, H-2-β). The location of other glucose proton resonances, comparatively upfield proved the absence of acylation at all the other glucose hydroxyl groups except numbers 2 and 3. These and the above given data (specifically results of controlled acid hydrolysis) would suggest a 3-*O*-galloyl-2-*O*-dehydrodigallic acid monocarboxyloyl-(α/β)-glucose structure for **9**. A reversed substitution, would produce on controlled hydrolysis a 3-*O*-dehydrodigallic acid monocarboxyloyl glucose as it is well known that an ester linkage at the glucose 2-OH is more labile and more easily hydrolyzed than the ester linkage at position no. 3 [4]. This was confirmed by the measurement of a long range shift correlation, HMBC spectrum of **9**, which revealed two cross peaks correlating the galloyl carbonyl carbon resonances in each of the α- and β-anomer to the resonances of the glucose H-3 proton resonances. Cross peaks correlating to the dehydrodigallic acid monocarboxyloyl carbonyl carbon signals in both anomers to the resonances of the H-2 glucose protons were also recognized. The minor ^2J correlations between H-2 and the etherified C-3 carbon and the detectable ^3J cross peaks correlating H-6′ to the free carboxylic carbonyl carbon in the dehydrodigalloyl moiety lent further support to the structure of **9** as 2-*O*-dehydrodigallic acid monocarboxyloyl-3-*O*-galloyl-(α/β)-glucose. The ^{13}C NMR data of **9** further confirmed its achieved structure. The α- and β-anomers were recognized, in the recorded spectrum from the resonances at δ 89.79 and 94.58 ppm, respectively. The acylation by dehydrodigallic acid mono-carboxyloyl and galloyl of the glucose OH groups at positions 2 and 3 follows from the upfield shift of the resonances of glucose C-1 and C-4 compared to the corresponding resonances in free D-glucopyranose [6f)]. These β-effects are in agreement with those reported for similarly substituted glucopyranose [6f)]. The upfield shifts of

the resonances of C-2 and C-3 are caused by both α- and β-effects which brought these resonances to δ ppm 72.07 (C-2-α), 71.69 (C-3-α), 73.25 (C-2-β), and 77.08 (C-3-β). The presence of only one dehydrodigallic acid monocarboxyloyl moiety and one galloyl moiety in **9** follows from the four carbonyl carbon resonances of the dehydrodigallic acid monocarboxyloyl moiety (two for each anomer) and the two galloyl carbonyl carbon (one for each anomer) resonances at δ ppm 164.42, 163.82, 166.88, and 167.37 (dehydrodigallic acid monocarboxyloyl C═O) and at 166.86, and 167.45 (galloyl C═O). Esterification of only one of the carboxyl groups of the dehydrodigallic acid monocarboxyloyl moiety followed from the recognizable upfield shift of the esterified carbonyl (δ 164.42, 163.82 in the α- and β-anomers). Assignment of the remaining carbon resonances were aided by comparison with the previously reported data of analogous compounds [6k)]. Furthermore, the measured δ values of the glucose carbon resonances confirmed that the sugar core exists in the pyranose form. Consequently, **9** is a 2-*O*-dehydrodigallic acid monocarboxyloyl-3-*O*-galloyl-(α/β)glucopyranose, which has not been reported previously in nature.



Compound **9**: 2-*O*-dehydrodigallic acid monocarboxyloyl-3-*O*-galloyl-(α/β) glucopyranose.

Compound **13** was isolated as an amorphous off-white powder which possesses galloyl ester-like characters (intense blue color with $FeCl_3$, rosy red color with KIO_3) [22] and UV spectral maximum in MeOH at 224, and 278 nm. Complete acid hydrolysis of **13** (2 N aq. HCl, 100 °C, 5 h) yielded glucose (CoPC), and gallic and dehydrodigallic acids. Both acids were individually isolated pure through MCI gel column fractionation of an EtOAc extract of the hydrolysate, using *n*-BuOH water saturated for elution and were identified by CoPC, ^1H and ^{13}C NMR (see Experimental). Controlled aqueous hydrolysis of **13** (H_2O, 100 °C, 12 h) yielded among other minors, a major intermediate, **13a**, (2DPC), separated pure by MCI-gel (CHP-20P) column fractionation of the dried aq. hydrolysate and elution with H_2O/MeOH mixtures of decreasing polarities. **13a** was identified to be 2-*O*-dehydrodigallic monocroxyloyl-3-*O*-galloyl-(α/β)glucopyranose through CoPC, ^1H and ^{13}C NMR. The positive ion FTESIMS spectra of **13** exhibited a molecular ion at m/z=1139 $[M+Na]^+$, corresponding to an *Mr* of 1116 Da. The accurate MSMS analysis of that ion revealed a fragmentation pattern consistent with the proposed structure. The ions found are: m/z: 969.1151 $[M+Na^+$-gallic acid-H$]^+$, m/z 825.0729 $[M+Na$-314$]^+$ which is due to breaking of the bonds of oxygen at C-1‴ and C-2‴, m/z 843.0712 $[M+Na$-314-O$]^+$ (breaking of the bonds at C-1‴ and C-14‴), m/z 657.0679 $[M-Na^+$-galloyl-dehydrodigalloyl glucose$]^+$ (breaking of the bonds at C-9″and C-14‴), m/z 505.0580 and m/z 487.0475 are due to breaking of the bonds at C-9″ and C-5‴ or C-9‴, respectively, thus suggesting a molecular structure consisting of two glucose, two dehydrodigallic acid dicarboxyloyl and one galloyl moieties incorporated together in the dimeric cyclic structure given for vermiculatin. The strong affinity to sodium (all fragments bear the sodium ion) adds evidence to the cyclic structure which looks like a cage compound. Structure elucidation of **13** was achieved by ^1H and ^{13}C NMR spectroscopy, including ^1H–^1H COSY, DEPT, HMQC and HMBC spectroscopy, which allowed the full assignment of all carbon and proton resonances. The ^1H, ^{13}C NMR and HMBC spectra (Table 1) unambiguously identified **13** as an ellagitannin with two dehydrodigallic dicarboxyloyl moieties whose four carbonyl resonances (HMBC) were located at δ ppm 164.20, 163.91, 163.80, and 163.73. This upfield location (in comparison with the corresponding resonances in the spectrum of free dehydrodigallic acid, is obviously due to esterification with the alcoholic glucose groups [21]. The presence of the galloyl moiety in the molecule of **13** was concluded from its characteristic resonances in the ^1H and ^{13}C NMR spectra (Table 1). The number and characteristic shifts of the ^{13}C sugar signals indicated the presence of two glucose systems in the pyranose form. The assignment of their ^1H and ^{13}C signals followed directly from the COSY and HMQC spectra. In all cases the ^{13}C shifts of their C-6 and C-4 signals indicated that these positions were un-substituted. This was also the case with one of the C-3 signals. All sugars had β-glycosidic linkages from the magnitude of the vicinal proton couplings of the anomeric protons in the ^1H spectrum. That one of the glucose moiety has esterified OH-1 and OH-2 groups, while the second glucose moiety has its OH-1, OH-2 and OH-3 groups esterified was readily identified from the low field shift of the geminal proton resonances of these OH groups [glucose-I: 4.80 (*d*, J=8.5 Hz, H-1), 4.75 (*dd*, J=8.5 and 9.5 Hz, H-2); glucose-II: 4.96 (*d*, J=8.5 Hz, H-1‴), 5.01 (*dd*, J=8.5 and 9.5 Hz, H-2‴), 5.29 (*t*, J=9.5 Hz, H-3‴)] and unambiguously confirmed by a long-range correlation between this proton and the carbonyl carbons C-7‴, C-14″, C-7″, C-14‴ and C-7⁗, respectively, in the HMBC spectrum. This latter spectrum also allowed the interconnectivity of the two glucoses, the two dehydrodigallicdicaboxyloyl and the galloyl units to be unambiguously determined. Proton H-1 (δ 4.80) showed a correlation with CO-7‴ (δ 163.80), H-2 (δ 4.75) with CO-14″ (δ 163.91), H-1‴ (δ 4.96) with CO-7″ (δ 163.73), H-2‴ (δ 5.01) with CO-14‴ (164.20) and H-3‴ with CO-7⁗(166.37). Similarly for the assignments of the aromatic carbon moieties a long-range correlation between H-2″, H-2‴ (δ 7.02 and 7.05), H-6″, H-6‴ (δ 6.02 and 5.99) and H-13″, H-13‴ (δ 7.22 and 7.16) in the two dehydrodigallic acid carboxyloyl moieties and H-1⁗ and H-5⁗ in the galloyl moieties to the carbons existing in the corresponding rings (see Table 1) was observed. Hence,



Compound **13**: vermiculatin.

the structure of **13** was determined as a dimeric cyclic ellagitannin, which we named vermiculatin.

In addition, the known compounds, 2,6-digalloyl glucose (**1**), kaempferol 3,7-disodium sulfate (**3**), quercetin 3,7-disodium sulfate (**5**), gallic acid (**6**), 3-mono methoxy ellagic 4-sodium sulfate (**7**), nilocitin (**8**), tamarixellagic acid (**10**), dehydrodigallic acid (**11**), decarboxy dehydrodigallic acid (**12**), quercetin (**14**), kaempferol (**15**), tamarixetin (**16**), ellagic acid (**17**), ellagic acid 3-monmethyl ether (**18**), ellagic acid 3,3′-dimethyl ether (**19**), and 6-hydroxy7-methoxycoumarine (**20**) were also isolated and identified by applying the conventional and spectral methods of analysis.

3.1. Antioxidant activity

3.1.1. DPPH assay

The radical scavenging activity of the crude extract was first determined in the DPPH assay. The IC_{50} of the extract is 5.8 μg/ml which is in a similar range as that for the positive control ascorbic acid with 1.8 μg/ml.

3.1.2. ORAC assay

Because of this high activity the antioxidative capacity was furthermore investigated in the ORAC assay. It demonstrated a moderate anti-oxidant capacity of the crude extract and higher activities of the isolated compounds **9** and **13** (Fig. 1).

3.2. Cytotoxicity: non tumorigenic cells

The *R. vermiculata* crude extract diminished the viability of the non tumorigenic HaCaT cells only in concentrations higher than 3.1 μg/ml and only in a moderate degree. In opposite, a slight increase in HaCaT cell viability was observed at the lowest concentration tested. There is also a moderate dose dependent cytotoxic effect in the higher concentrations. The higher bar seen at a concentration of 25, 50 and 100 μg/ml for compound **13** and at a concentration of 100 μg/ml for compound **9** is probably not due to an increased cell viability but rather to undissolved substance particles (Fig. 2). Cell viability due to tested concentrations of all test samples was well above the IC_{50} limit, so the latter could not be estimated. The positive control led to an increased cell viability of about 150%. The results of the biological investigation of the extract and two of the isolated novel compounds confirm the antioxidant potential of plant phenolics. The cytotoxicity is neglectable, and small concentrations of the extract have yet a stimulating effect on the investigated skin cells. The results substantiate the ethnomedicinal use of *R. vermiculata*.

3.3. Cytotoxicity against tumor cell lines

SRB-U assay was used to assess the cytotoxicity of the *Reaumuria* extract against four different solid tumor cell lines. The cytotoxicity parameters, IC_{50} and R-fraction were calculated using the E_{max} model as described in the Biological methods section. The *Reaumuria* extract showed comparable potencies against all solid tumor cell lines used with IC_{50} ranging from 1.3 ± 0.15 to 2.4 ± 0.22 μg/ml (Table 2). The cytotoxicity pattern (dose–response profile) of *Reaumuria* extract was sharp in HCT-116, MCF-7 and PC-3 cell lines (Fig. 3-A) with a relatively

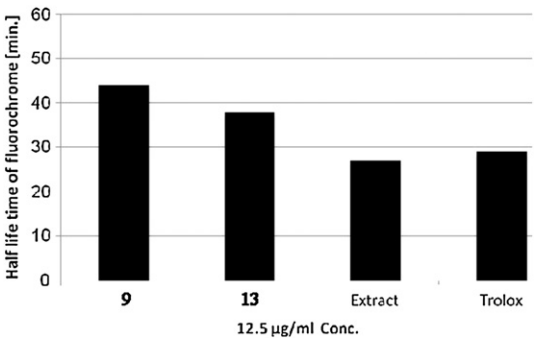


Fig. 1. Antioxidant activity of *R. vermiculata* samples measured in the ORAC assay: half life of fluorescein after protection with 12.5 μg/ml test sample. The positive control is Trolox (3.13 μM).

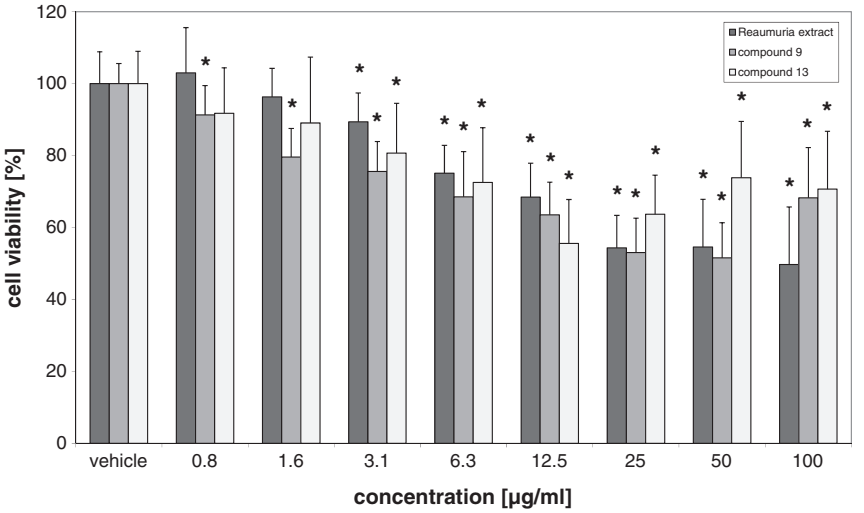


Fig. 2. Viability of HaCaT keratinocytes after treatment with *Reaumuria* extract, 2-*O*dehydrodigallicacid monocarboxyloyl-3-*O*-galloyl-(α/β)glucopyranose (**9**) or vermiculatin (**13**) measured in the MTT assay. Experiments were carried out in 4 independent experiments with 6 replicates.

higher Hill-type co-efficient ranging from 4.22 to 8.47. On the other hand, the dose response pattern was gradual with relatively lower Hill-type co-efficient (2.38) in Huh-7 liver cancer cell line. *Reaumuria* extract possesses the highest IC_{50} in Huh-7. However, the resistant fraction of Huh-7 was the lowest (0%) among all tested cell lines. Other cell lines showed substantial R- fraction ranging from 0.88±0.2 to 2.4±0.6 (Table 2). The types of cancers examined herein were selected based on clear epidemiological evidence and high health related problem. Liver cancer constitutes a national health problem in Egypt and Middle East. Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide. Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males [23]. The observed IC_{50}s of the *Reaumuria* extract represents a promise for universal potencies against different types of cancers with high deteriorating health damage.

According to the cytotoxic profile of the crude extract, promisingly low IC_{50} and R-fraction in addition to a reasonable value of hill type co-efficient were noticed in the case of prostate cancer cell line (PC-3) which warranted further investigation. The very high value of hill-type co-efficient suggests non specificity in cytotoxic molecular target and vice versa [24,25]. Compounds **9** and **13** were therefore, tested against the prostate cancer cell line. The cytotoxicity pattern (dose–response profile) of both compounds in PC-3 cell was much gradual than that of the total extract (Fig. 3-B) with hill-type co-efficients of 0.5 and 0.8, respectively. Compounds **9** and **13** possess IC_{50}s of 1.5±0.33

Table 2
Cytotoxicity of *Reaumuria* extract against different solid tumor cell lines.

Cell line	Tumor origin	Cytotoxicity parameter		
		IC_{50} (µg/ml)	R-fraction (%)	Hill-type co-efficient
Huh-7	Liver	2.4±0.22	0.0±0.0	5.59
HCT-116	Colorectal	1.8±0.94	2.2±0.58	2.38
MCF-7	Breast	1.3±0.15	0.88±0.2	8.47
PC-3	Prostate	1.5±0.17	2.4±0.6	4.22

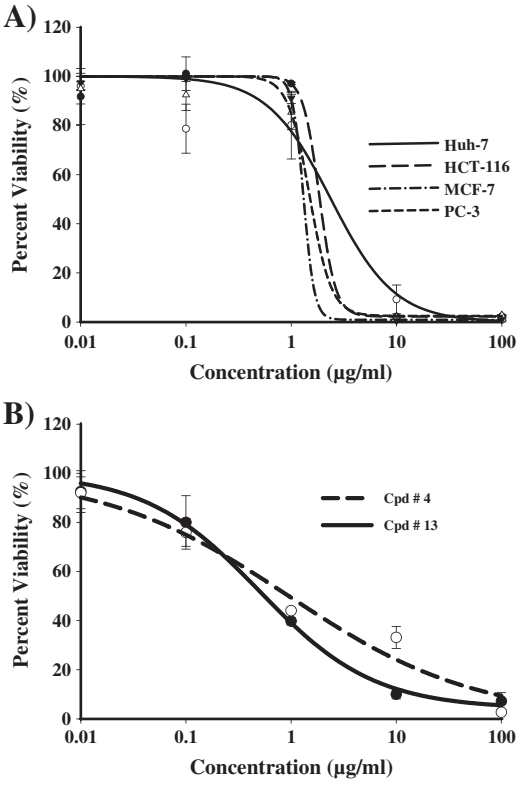


Fig. 3. Dose–response curve of *Reaumuria* extract and its derived purified compounds against solid tumor cell lines. Huh-7 (○), HCT-116 (●), MCF-7 (▼), and PC-3 (Δ) cells were exposed to the crude extract for 72 h (A). PC-3 cell line was exposed to **9** (○) and **13** (●). Cell viability was determined using SRB-U assay and data are expressed as mean±S.D. (n=3).

Table 3
Cytotoxicity of **9** and **13** of the *Reaumuria* extract against prostate cancer cell lines (PC-3).

Compound	Cytotoxicity parameter		
	IC_{50} (μM)	R-fraction (%)	Hill-type co-efficient
9	1.5±0.33	0.0±0.0	0.49
13	0.45±0.09	4.1±0.94	0.8

and 0.54±0.09μM, respectively and resistant fractions of 0.0% and 4.1%, respectively (Table 3).

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