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Polyphenols in *Ammania auriculata*: Structures, antioxidative activity and cytotoxicity

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Chemical and biological investigations of the extract of *Ammania auriculata* (Lythraceae) resulted in the identification of eight polyphenols (**1** – **8**) for the first time from this plant, including the gallotannin, 2,3,6-tri-*O*-galloyl-(α,β)-⁴C₁-glucopyranose (**8**), for which 1D and 2D-NMR spectra were recorded and assigned for the first time. The structures of all isolates (**1** – **8**) were elucidated by conventional methods, spectroscopic analysis, including 1D and 2D NMR, and by HR-ESIMS as well. All of the isolated compounds were evaluated for their antioxidant activities, determined by the DPPH and ORAC methods and for their cytotoxicity against the keratinocyte cell line HaCaT using the neutral red assay (NRU) and cell cycle analysis. Compounds **1**, **3**, **4**, **5**, and **6** significantly inhibited reactive oxygen species production with ED₅₀ values between 3.22 and 9.79 μ g/ml. Compounds **1**, **3**, **4**, and **5** showed cytotoxic activity against HaCaT cells with IC₅₀ values between 30.7 and 84.1 μ g/ml. The new galloyl glucose (**8**) was found not cytotoxic. Ellagittannins, 2,3-hexahydroxy-(α,β)-glucopyranose (**1**) and 1-*O*-galloyl 2,3-hexahydroxy-(α)-glucopyranose (**5**) possess remarkable antioxidative and comparably weak cytotoxic activity.

1. Introduction

In Egypt, several plants rich in phenolics have been investigated for their biological activities and their constitutive phenolics (e.g. Nawwar et al. 2011, 2012a, 2012b, 2013) as well. Among those plants, almost nothing is known about the phytochemistry of *Ammania auriculata* Willd., synonym: *Ammannia arenaria* Kunth., apart from a single report describing the presence of two oleananes, kaempferol, quercetin, kaempferol-3-*O*- α -L-arabinofuranoside, kaempferol-3-*O*- β -D-xylopyranoside and ellagic acid in aerial parts of the plant (Gohar et al. 2012). *A. auriculata* is used as a counter irritant for rheumatic pains (Parekh and Chanda 2007). It belongs to the Lythraceae family which has 31 genera arranged in five subfamilies (Taeckholm 1974). During the current study we isolated and identified eight phenolics (**1** – **8**) from the ethanol extract of whole *A. auriculata* plant. The isolates are characterized for the first time from that plant. They include the new natural phenolic, 2,3,6-tri-*O*-galloyl-(α,β)-glucopyranose (**8**). Also, we evaluated the antioxidant capacities using the DPPH and the ORAC method and assessed the cytotoxicity against the keratinocyte cell line HaCaT using the neutral red assay (NRU) and cell cycle analysis for that extract and all of its isolated compounds.

2. Investigations, results and discussion

Specimens of the dried flowering whole *Ammania auriculata* plant were exhaustively extracted with aqueous ethanol. The

received extract was subjected to a series of column and preparative paper chromatographic separations to isolate compounds **1** – **8**. Compound **8** has not been described before.

2.1. Structure elucidation

Compound **8** was isolated as an off-white amorphous powder. Its chromatographic properties, color reactions (dark blue with FeCl₃, rose color with KIO₃) and UV spectral data (one band at 275 nm) were consistent with those of gallotannins. It exhibited a molecular ion at [M – 1][–]: m/z = 635.4616, corresponding to a molecular formula of C₂₇ H₂₃ O₁₈, (calc.: 635.461795) in negative HRESI-MS. After normal acid hydrolysis, **8** yielded glucose, and gallic acid (CoPC) and on controlled acid hydrolysis it yielded an intermediate, **8a**, which was extracted from the aqueous hydrolysate by ethyl acetate and purified over Sephadex LH-20 column. **8a** was fully identified through CoPC, UV, HRESI-MS, ¹H and ¹³C NMR to be 3,6-digalloyl glucose (Nawwar and Hussein 1994). Consequently, **8** is the monogalloyl derivative of **8a**. To find out the site of attachment of the galloyl moiety to the 3,6-digalloyl glucose moiety to form **8**, ¹H NMR spectral analysis was then engaged. The spectrum revealed two distinct patterns of proton signals belonging to substituted α - and β -glucose anomers. Each pattern was found to contain well separated signals of the seven-spin system belonging to a distinct glucose anomer. The spectrum also showed three pairs of singlets in the aromatic region for the three galloyl moieties (three

singlets for each anomer). The appearance of two signals for each distinct proton in **8** proved the presence of a free anomeric glucose hydroxyl group, thus restricting the site of attachment of the galloyl moiety to either the glucose position number 2 or to number 4 in the 3,6-digalloyl glucose moiety. The ambiguity in determining the site of attachment between the galloyl and the 3,6-digalloyl glucose moieties to form **8** was then unraveled through extensive NMR analysis: 1D- ^1H , ^{13}C , 2D-COSY, HSQC and HMBC. The correlations recognized in the ^1H - ^1H COSY spectrum together with the coupling constants measured from the 1D- ^1H NMR spectrum allowed the definition of the glucose protons in both the α - and β - anomers as follows: 5.37 (*d*, 5 = 3.5 Hz, H-1- α), 4.95 (*dd*, *J* = 8 and 3.5 Hz, H-2- α), 5.70 (*t*, 5 = 8 Hz, H-3- α), 3.70 (*m*, H-4- α), 3.95 (*m*, H-5- α), 4.38 (*d*, *J* = 12.5 Hz, H-6- α), 4.25 (*dd*, *J* = 12.5 Hz and 4.5 Hz, H-6'- α); β -glucose moiety: 5.05 (*d*, *J* = 8 Hz, H-1- β), 5.10 (*t*, *J* = 8 Hz, H-2- β), 5.33 (*t*, *J* = 8 Hz, H-3- β), 3.8 (*m*, H-4- β), 3.92 (*m*, H-5- β), 4.42 (*d*, *J* = 12.5 Hz, H-6- β), 4.29 (*dd*, *J* = 12.5 and 4.5 Hz, H-6'- β). The location of the H-2 glucose proton signals downfield at δ 4.95 (H-2- α) and 5.10 (H-2- β) in comparison to their location in the ^1H NMR spectrum of 3,6-digalloylglucose at δ 3.5-3.9 in both anomers confirmed galloylation of their geminal OH group (number 2). The weight of evidence given above, proved that compound **8** is 2,3,6-tri-*O*-galloyl-(α/β)- $^4\text{C}_1$ -glucose. ^{13}C NMR spectral analysis of **8** afforded a spectrum containing double signals for most of the glucose and galloyl carbons. Resonances were assigned by comparison with the ^{13}C NMR data, reported for similar galloyl glucoses, as well as by consideration of the known α - and β -effect (Nawwar et al. 1984) caused by esterifying the sugar hydroxyl groups and was confirmed by measuring HSQC spectrum. Attachment of galloyl moiety to C-2 of glucose was evidenced by the β -upfield shift recognized for the vicinal anomeric carbon resonances, as well as for the resonance of C-3 (all in comparison with the chemical shifts of the corresponding carbon resonances in the spectrum of unsubstituted glucopyranose). In both anomers, C-2 was found to resonate upfield (α -effect) at 72.2 (C-2- α) and 73.5 (C- β). The measured chemical shift values of the glucose carbon resonances proved that this moiety existed in the pyranose form, thus confirming the final structure of **8** to be 2,3,6-tri-*O*-galloyl-(α/β)- $^4\text{C}_1$ -glucopyranose (Fig. 1).

2.2. Known compounds

2,3-Hexahydroxy-(α/β)-glucopyranose (**1**), 2,3-di-*O*-galloyl-(α/β)-glucopyranose, nilocitin (**2**), 1,6-di-*O*-galloyl-(α/β)-glucopyranose (**3**), kaempferol 3-*O*-(6''-para-coumaroyl-glucopyranoside) (**4**), 1-monogalloyl-2,3-hexahydroxy-(α)-glucopyranose (**5**), quercetin 3-*O*-(6''-galloyl-glucopyranoside) (**6**), gallic acid 3-*O*- β -glucoside (**7**).

2.3. Biological activities

In order to get information about biological activities of the extract of *A. auriculata* and some of the isolated substances the samples were tested for antioxidant activity and cytotoxicity. The influence of the whole extract and three of the isolates on the viability of HaCaT keratinocytes was tested in neutral red assay (IC_{50} values in Table 1). The vehicle in which the test examples were dissolved had no influence on viability of HaCaT cells. Using etoposide (positive control for cytotoxicity) viability of HaCaT cells was reduced to 40 to 60%. 2,3-Di-*O*-galloyl- α/β -glucopyranose, nilocitin (**2**) was more cytotoxic than the whole extract or gallic acid 3-*O*-glucoside (**7**). Kaempferol 3-*O*-(6''-p-coumaroyl- β -glucopyranoside) (**4**) and the novel compound

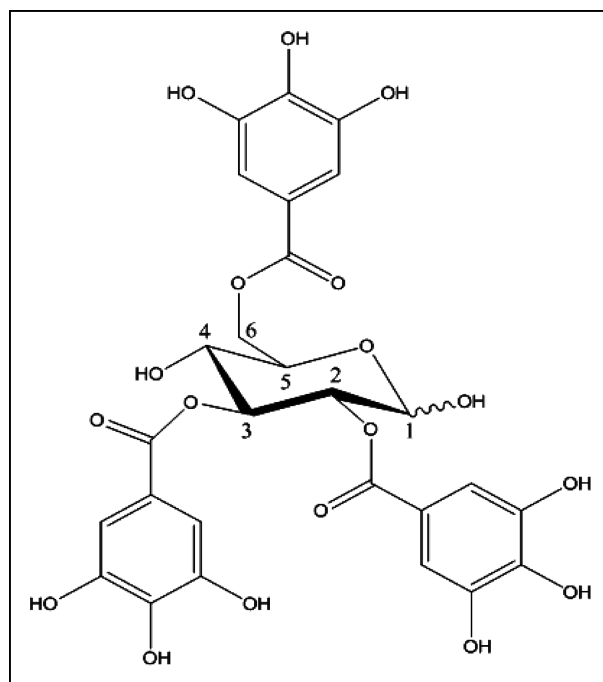


Fig. 1: Chemical structures of a 2,3,6-tri-*O*-galloyl-(α/β)- $^4\text{C}_1$ -glucopyranose (**8**).

2,3,6-tri-*O*-galloyl-(α/β)-glucopyranose (**8**) did not show any cytotoxicity against the HaCaT cells.

Antioxidant activity was tested by the ORAC assay (Brand-Williams and Cuvelier 2012) and for the whole extract also by the DPPH assay (Brand-Williams and Cuvelier 2012; Mishra et al. 2012). In case of early fluorescence reduction, antioxidative activity is low as shown for 2,3-di-*O*-galloyl-(α/β)-glucopyranose (**2**) in concentrations below 1.95 $\mu\text{g}/\text{ml}$. Higher concentrations resulted in better antioxidant activity (Fig. 2). Table 1 summarizes the antioxidant activity (ED_{50}) as determined by the ORAC assay and cytotoxicity (IC_{50}) of the whole extract compared to eight isolates. For the whole extract an antioxidant activity with an ED_{50} value of $14.5 \pm 3.2 \mu\text{g}/\text{ml}$ was detected. This ED_{50} was confirmed using the DPPH assay with $13.3 \pm 5.6 \mu\text{g}/\text{ml}$ (value for the positive control ascorbic acid 1.83 $\mu\text{g}/\text{mL}$). Since for the DPPH assay high amounts of substances are required and the results between both methods were comparable, we continued the investigations of isolated compounds with the ORAC assay only. In any case the IC_{50} values for cytotoxicity were higher than the ED_{50} for antioxidant activity (Table 1).

To get more insight into cytotoxic activities of the extract and six of the isolated compounds cell cycle progression was inves-

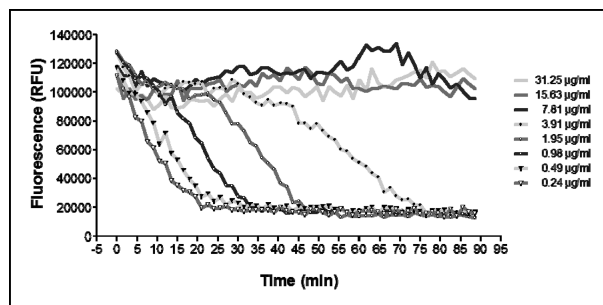


Fig. 2: Representative example of radical scavenging activity of nilocitin [2,3-Di-*O*-galloyl-(α/β)-glucopyranose (**2**)] measured by ORAC assay. Concentration tested ranged from 31.25 to 0.24 $\mu\text{g}/\text{ml}$. Relative fluorescence was constant over the whole measurement period up to a concentration of 7.81 $\mu\text{g}/\text{ml}$ demonstrating high antioxidant activity with an ED_{50} of 4.95 $\mu\text{g}/\text{ml}$.

Table 1: Cytotoxicity (IC₅₀) and radical scavenging activity (ED₅₀) of isolated compounds in comparison to the extract of *Ammania auriculata*

	Cytotoxicity [IC ₅₀ (μg/ml)]	Radical Scavenging Activity [ED ₅₀ (μg/ml)]
Extract of <i>Ammania auriculata</i>	106.51 ± 10.22	14.53 ± 3.32
2,3-HHDP-(α/β)-glucopyranose (1)	84.09 ± 0.65	9.79 ± 0.57
2,3-Di- <i>O</i> -galloyl-(α/β)-glucopyranose (2)	30.68 ± 8.72	4.95 ± 0.82
1,6-Di- <i>O</i> -galloyl-(α/β)-glucopyranose (3)	39.05 ± 8.49	3.22 ± 1.76
Kaempferol 3- <i>O</i> -β-(6''-p-coumaroyl-β-glucopyranoside) (4)	> 500	6.68 ± 0.54
1-Monogalloyl 2,3-HHDP-α-glucopyranose (5)	82.09 ± 3.55	7.14 ± 0.62
Quercetin 3- <i>O</i> -β-(6''-galloyl-β-glucopyranoside) (6)	71.80 ± 15.35	5.56 ± 0.87
Gallic acid 3- <i>O</i> -β-glucoside (7)	184.20 ± 6.30	n.d.
2,3,6-Tri- <i>O</i> -galloyl-α/β-glucopyranose (8)	> 250	n.d.

Results are given in mean ± SD of 3 independent experiments

tigated after the HaCaT cells were cultured for 24 h or 72 h in the presence of each one of the test samples (Fig. 3 and Table 2). After 24 h incubation only 2,3-HHDP-(α/β)-glucopyranose (1) influenced the cell cycle, namely a significant G2/M arrest. Incubation of HaCaT cells for 72 h with 2,3-HHDP-(α/β)-glucopyranose (1) and 2,3-di-*O*-galloyl-(α/β)-glucopyranose, nilocitin (2) resulted in a G2/M and S arrest at the expense of the G1 phase of cell cycle (Fig. 2 and Table 2). Cells in the SubG1 phase, a sign for cells in apoptosis, were never detected.

3. Experimental

3.1. General

NMR spectra were acquired in DMSO-*d*₆ on a Jeol ECA 500 MHz NMR spectrometer, at 500 MHz. Standard pulse sequence and parameters were used to obtain one-dimensional ¹H and ¹³C, and two dimensional COSY, HSQC and HMBC spectra. ¹H chemical shifts (δ) were measured in ppm, relative to TMS and ¹³C NMR chemical shifts to acetone-*d*₆ and were converted to TMS scale by adding 29.8. High resolution ESI mass spectra were measured using a Finnigan LTQ FT Ultra mass spectrometer (Thermo FisherScientific, 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. UV recording was made on a Shimadzu UV–Visible-1601 spectrophotometer. Chromatographic analysis (PC) was carried out on Whatman No. 1 paper, using solvent systems: (1) H₂O; (2) 6% HOAc; (3) BAW (n-BuOH–HOAc–H₂O, 4:1:5, upper layer)

3.2. Plant material

Flowering whole plants of *Ammania auriculata* were collected from the rise fields near Banha town, forty km north Cairo, Egypt, in April, 2012. A flowering voucher specimen (A 362) is deposited in the herbarium of the National Research Center (NRC), the identity of which was verified by Dr. S. Kwashty, Prof. of Botany at NRC.

3.3. Preparation of extract

Ground air dried flowering whole plant samples (3 kg) were extracted (thrice) with 5 l EtOH/H₂O (3:1) under reflux for eight hours each. The extract was filtered and dried in vacuum to yield a dark brown amorphous powder (250 g).

3.4. Isolation and identification of phenolics

115 g of the dried aqueous EtOH extract was loaded onto a Sephadex LH-20 (900 g) column (120 × 7.5 cm). Elution then started with H₂O followed by isocratic elution in 10 % steps from 10 % to 100 % MeOH. Following removal of the solvents ten fractions (I – X, fraction I eluted with H₂O; II with 10 %, III with 20 %, IV with 30 %, V with 40 %, VI with 50 %, VII with 60 %, VIII with 70 %, IX with 80 % and X with 90 %) were individually collected and subjected to two dimensional paper chromatography (2DPC). Compound 1 (108 mg) was isolated pure from 2.05 g of fraction II by repeated precipitation (thrice) from acetone by ether. Compounds 2 (104 mg) and 3 (75 mg) were individually separated pure by fractionation of 2.3 g of fraction IV over Sephadex LH-20 (15 g) column using a MeOH/H₂O mixture of decreasing polarity for elution. Compound 4 (92 mg) was individually isolated pure from 679 mg of fraction V by fractionation on a Sephadex LH-20 (13 g) column and 40% aqueous MeOH for elution, followed by preparative paper chromatography (prep. PC) using BAW for final purification. Compound 5 (94 mg) was individually separated pure from 701 mg of fraction VI by prep. PC, using n-BuOH water saturated as solvent. Compound 6 (75 mg) was individually obtained pure from 612 mg of fraction VII through repeated prep PC (thrice), using BAW for elution. Extraction of 1.7 g of fraction X with ether (150 ml, 3 times, each for 1 h, under reflux) followed by filtration and removal of ether from the filtrate under vacuum, afforded 800 mg of yellow material. The dried ether insoluble material (542 mg) thus left was applied on the top of MCI gel column, using MeOH/H₂O mixtures of decreasing polarities for elution to afford pure individual samples of compound 7 (45 mg) and 8 (84 mg).

2,3,6-Tri-*O*-galloyl-(α,β)-⁴C₁-glucopyranose (8): *R*_f-values: 0.59 (H₂O), 0.42 (HOAc), 0.34 (BAW); UV λ_{max} (nm) (log ε) in MeOH: 275 (3.86); [α]_D³⁰ + 10.9° (acetone; *c* 0.6). ESI-MS, negative mode, [M–H][–] = 635; HRESI-MS, negative mode: *m/z* = 635.4616, corresponding to a molecular formula of C₂₇ H₂₃ O₁₈ (calc.: 635.4618). On complete acid hydrolysis (14 mg in 20 ml aq. 1.5 N HCl. 100°. 5 hrs) compound 8 yielded glu-

Table 2: Summary of the influence of the whole *Ammania auriculata* extract and six isolates on the cell cycle of HaCaT cells

	24 h	Cycle of HaCaT Cells 72 h
Extract of <i>Ammania auriculata</i>	no influence	n.d.
2,3-HHDP-(α/β)-glucopyranose (1)	G2/M ↑↑	G1 ↓↓↓↓, G2/M ↑↑, S ↑↑↑↑
2,3-Di- <i>O</i> -galloyl-(α/β)-glucopyranose (2)	no influence	G1 ↓↓↓, G2/M ↑↑↑, S ↑↑↑
1,6-Di- <i>O</i> -galloyl-(α/β)-glucopyranose (3)	no influence	n.d.
Kaempferol 3- <i>O</i> -β-(6''-p-coumaroyl-β-glucopyranose) (4)	G1 (↑)*	n.d.
1-Monogalloyl 2,3-HHDP-(α)-glucopyranose* (5)	no influence	n.d.
Quercetin 3- <i>O</i> -β-(6''-galloyl-β-glucopyranoside) (6)	no influence	n.d.

Cells were incubated at concentrations which corresponded to the IC₅₀ values. *only n = 1 due to limited amount of this isolate. Significance was checked by One-way Anova with Tukey's Multiple Comparison test: significant increase ↑ (p < 0.05), ↑↑ (p < 0.01), ↑↑↑ (p < 0.001); significant reduction ↓↓ (p < 0.01), ↓↓↓ (p < 0.001); n.d. = not determined

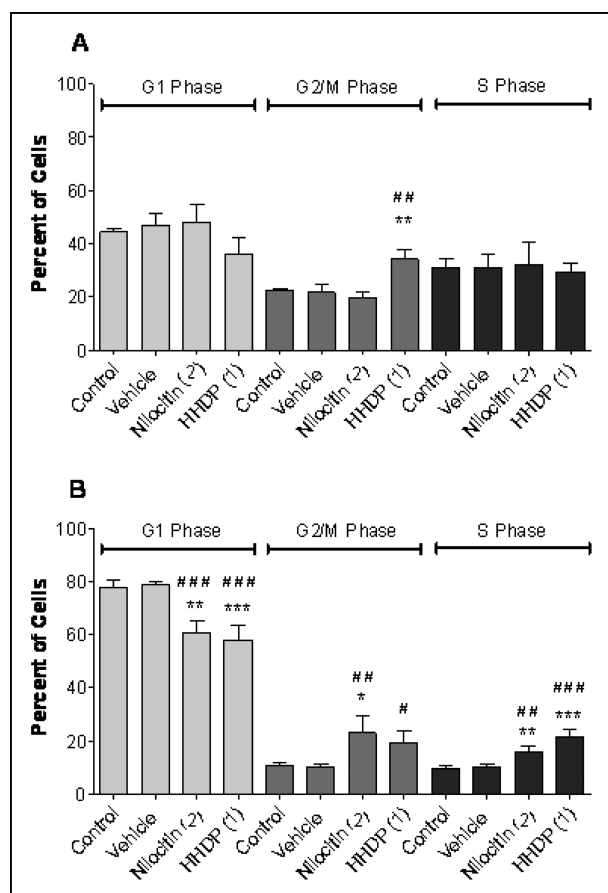


Fig. 3: Cell cycle of HaCaT keratinocytes determined 24 h (A) and 72 h (B) after culture of cells in the presence of 2,3-digalloyl-*O*-(α/β)-glucopyranose (2) [nilocitin] or 2,3-HHDP-(α/β)-glucopyranose (1). Cells were incubated at concentrations which corresponded to the IC₅₀ values of the isolates. Results are given as mean $\pm$ SD of 3 independent experiments. Significance was checked by One-way Anova with Tuckey's Multiple Comparison test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ vs. vehicle.

cose (CoPC) and gallic acid (CoPC and ^1H NMR). On controlled acid hydrolysis (41 mg in 15 ml aq. 0.5 N HCl, 100°C. 2 hrs) it yielded 3,6-di-*O*-galloyl-(α/β)-glucose (**8a**). Compound **8a**: *R*. values: 62 (H_2O), 69 (AcOH), 43 (BAW). UV data: λ_{max} (nm): 275. *M*_r 484; negative ESIMS, $[\text{M}-\text{H}]^- = 483$; HRESI-MS negative mode: $m/z = 483.079$, corresponding to a molecular formula of $\text{C}_{20}\text{H}_{19}\text{O}_{14}$ (calc. 483.3572). On complete acid hydrolysis of **8a** (7 mg, worked up as usual) it yielded gallic acid and glucose (CoPC). On controlled acid hydrolysis it (9 mg) yielded 6-monogalloyl glucose (CoPC). ^1H NMR of **8a**: α -glucose moiety: 5.01 (*d*, *J* = 3.5 Hz, H-1) 3.5-3.9 (m, H-2 and H-4), 5.3 (*t*, *J* = 9 Hz, H-3), 3.88 (m, H-5), 4.38 (*d*, *J* = 12.5 Hz, H-6), 4.25 (*dd*, *J* = 12.5 and 4.5 Hz, H-6'); β -glucose moiety: 5.05 (*d*, *J* = 9 Hz, H-1), 3.5-3.9 (m, H-2 and H-4), 4.98 (*t*, *J* = 9 Hz, H-3), 3.9 (m, H-5), 4.42 (*d*, *J* = 12.5 Hz, H-6), 4.29 (*dd*, *J* = 12.5 and 4.5 Hz, H-6'); galloyl moieties: 6.94 (*s*), 6.96 (*s*), 6.99 (*s*), 7.01 (*s*). $^1\text{HNMR}$ of **21**: α -glucose moiety: 5.37 (*d*, *J* = 3.5 Hz, H-1), 4.95 (*dd*, *J* = 8 and 3.5 Hz, H-2), 5.70 (*t*, *J* = 8 Hz, H-3), 3.70 (*m*, H-4), 3.95 (*m*, H-5), 4.38 (*d*, *J* = 12.5 Hz, H-6), 4.25 (*dd*, *J* = 12.5 Hz and 4.5 Hz, H-6'); β -glucose moiety: 5.05 (*d*, *J* = 8 Hz, H-1), 5.10 (*t*, *J* = 8 Hz, H-2), 5.33 (*t*, *J* = 8 Hz, H-3), 3.8 (*m*, H-4), 3.92 (*m*, H-5), 4.42 (*d*, *J* = 12.5 Hz, H-6), 4.29 (*dd*, *J* = 12.5 and 4.5 Hz, H-6'); galloyl moieties: 7.0 (*s*), 6.99 (*s*), 6.98 (*s*), 6.95 (*s*), 6.93 (*s*), 6.88 (*s*). ^{13}C NMR of **21**: α -glucose moiety: 91.2 (C-1), 72.2 (C-2), 72.8 (C-3), 69.3 (C-4), 72.3 (C-5), 64.3 (C-6); β -glucose moiety: 95.3 (C-1), 73.5 (C-2), 75.5 (C-3), 70.8 (C-4), 74.9 (C-5), 64.4 (C-6); galloyl moieties: 121.6, 121.7, 121.8 (C-1), 109.8, 109.9, 110.2 (C-2 and C-6), 146.1, 146.0, 145.9, 145.4 (C-3 and C-5), 139.3, 138.6, 138.8 (C-4), 167.0, 166.8, 166.6, 165.0 (C=O).

3.5. Biological assays

3.5.1. Radical scavenging effect

The estimation was done according to the method of Brand-Williams and Cuvelier (1995). DPPH (2,2-diphenyl-1-picrylhydrazyl), a stable radical, is reduced after reaction with an antioxidant compound and its absorbance at 517 nm is then reduced. The reaction mixture contained 500 μL of test

extract, 375 μL ethanol and 125 μL of a 1 mM freshly prepared DPPH solution in ethanol. Different concentrations of test samples were prepared while the final concentration of DPPH in the reaction mixture was 0.125 mM. After incubation of the mixture at 37°C for 30 min in the dark the absorbance was measured at 517 nm. Blank samples contained the same amount of methanol and DPPH solution. All experiments were carried out in triplicate. Ascorbic acid was used as positive control. Percentage radical scavenging activity of samples was calculated using the following formula:

$$\text{Radical Scavenging Activity(\%)} = \left[\frac{(A_{\text{Blank}} - A_{\text{Sample}})}{A_{\text{Blank}}} \right] 100$$

ED₅₀ values, the concentration of the substrate that causes 50% loss of the DPPH activity (colour), were calculated for the standard and the extract from a graph plotted for the % inhibition against the concentration in $\mu\text{g}/\text{mL}$.

3.5.2. Oxygen radical absorbance capacity

Reactive oxygen species, ROS are generated by the thermal degradation of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and quench the signal of the fluorescent probe fluorescein. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of the fluorochrome. A vitamin E derivative, 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), was used as positive control. Test compounds **5** and **6** were dissolved in phosphate buffered saline (10 mM, pH 7.4) and investigated for their antioxidant capacity. Experiments were done in black 96-well plates. In each well of a 96-well Plate 150 μL fluorescein (final concentration: 2.5 nM), 25 μL Trolox (final concentrations: 0.78 – 25 μM) or 25 μL test compound were pipetted in quadruplicate. Plate was allowed to equilibrate at 37°C for 30 min. After this time, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 90 s; first to determine the background signal. After three cycles 25 μL AAPH (final concentration: 60 mM) were added manually in each well with a multi-channel-pipette. This was done as quickly as possible since the ROS generator displays immediate activity after addition. Fluorescence measurements were continued for 90 min. Half life time of fluorescein was determined using MS Excel software.

3.5.3. Cytotoxicity assay

HaCaT cells were obtained from CLS Cell Lines Service (Eppenheim, Germany). Cells were cultured in RPMI 1640 medium (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 8 % fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) and antibiotics (100 U/ml penicillin/100 $\mu\text{g}/\text{mL}$ streptomycin; Sigma Aldrich, Taufkirchen, Germany) at 95% humidity, 5% CO₂ and 37°C. HaCaT cells were sub-cultured twice a week and regularly tested for mycoplasma contamination. Cytotoxicity of test samples against both cell lines was investigated using the neutral red uptake (NRU) assay. After 24 h cultivation in 96 well plates (3 or 8 $\times 10^3$ cells/well) medium was removed and cells were exposed for 72 h to various concentrations (max. 500 $\mu\text{g}/\text{mL}$) of test samples. After removal of the medium wells were washed with HBSS (Hanks Balanced Salt Solution, PAA). Cells were then incubated for 3 h with 100 μL 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red, Merck, Darmstadt, Germany, stock solution 3.3 $\mu\text{g}/\text{mL}$; working solution 33 ng/mL). Medium was removed and wells were washed twice with HBSS. Afterwards cells were lysed with 100 μL of 1% acetic acid in 50% EtOH. Finally, after 45 min optical density was measured at 450 nm in a plate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany). The IC₅₀ values were defined from obtained dose-response curves and expressed in mean $\pm$ SD. All compounds were tested in duplicate. Etoposide (Alexis Biochemicals, ≥ 98 % purity) was used as positive control.

3.5.4. Cell cycle analysis

The distribution of HaCaT cells in different cycle stages was analyzed using flow cytometry 24 h and 72 h after the cells were cultured in the presence of the extract of *A. auriculata* and six of the isolates. Briefly, 1×10^5 cells were seeded into 24 well plates with 500 μL RPMI 1640 medium, 8% FCS. After overnight culture at 37°C medium was removed and 500 μL extract or isolates (IC₅₀) were added. Control cells received 500 μL medium or vehicle. Cells were then further cultured for 24 h or 72 h. After culture the cells were detached and counted using a Neubauer chamber. 5×10^5 cells were incubated in 1 mL cold (−20°C) 70 % ethanol at least for 1 h (4°C) and washed twice with 2 mL PBS (without Ca²⁺/Mg²⁺). Subsequently, the cells were incubated for 30 min in RNase (50 $\mu\text{g}/\text{mL}$ in PBS) at 37°C. After washing with 2 mL FACS buffer (1 % FCS, 8 $\mu\text{g}/\text{mL}$ sodium azide in PBS), the cells were resuspended in 500 μL FACS buffer and stained with 25 μL propidium iodide (50 $\mu\text{g}/\text{mL}$). 5×10^3 cells per sample were analyzed with a MAC-

SQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Kaluza Software (Beckman Coulter, Krefeld, Germany) was used for evaluating the percentage distribution of HaCaT in each cell cycle phase.

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