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# A cytotoxic flavonol glycoside from *Melaleuca leucadendra* leaves extract with immunostimulant activity

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Received July 18, 2017, accepted September 15, 2017

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*Pharmazie* 73: 61–64 (2018)

doi: 10.1691/ph.2018.7785

Leaves of *Melaleuca leucadendra* contain the novel flavonol glycoside, myricetin  $3-O-\beta^-4C_1$ -galactopyranuronoid. In addition, known fifteen phenolics were identified. All isolates are characterized for the first time from this plant. Structures were established by conventional methods and confirmed by spectral methods of analysis, including one and two-dimensional nuclear magnetic resonance spectroscopy (1D and 2D-NMR) and high resolution electro-spray ionization mass spectrometry (HRESIMS), as well. Assessment of some immunological and biological efficacy, of the extract in combination with a parallel cytotoxicity evaluation, using the method of cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique was carried out. Besides, evaluation of the antioxidant effectiveness, using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the oxygen radical absorption capacity (ORAC) methods was performed. In addition, the cytotoxicity against liver (Huh-7), breast (MCF-7) and prostate (PC-3) cancers using the neutral red assay (NRU) technique for the extract and the new flavonol glycoside also, was assessed.

# 1. Introduction

During our ongoing search for novel bioactive metabolites from Melaleuca species (Hussein et al. 2007) the 75 % aqueous ethanol extract of the leaves of Melaleuca leucadendra (L.) L. was selected to be investigated due to its interesting phytochemical and biological properties. The extract displayed a rich content in phenolic metabolites as revealed by its high performance liquid chromatography-electrospray ionization mass spectra (HPLC-ESIMs) and two dimensional paper chromatographic (TDPC) profile as well as strong potential to promote in vivo and in vitro immune stimulant activity. Melaleuca leucadendra, the fine leafed paper bark, also known as weeping paper bark is a hardy native tree with bright green leaves, papery bark and creamy pale yellow flowers in spring/summer with a maximum height of 10 to 14 meters and a maximum spread of 8 to 10 meters. A range of antibacterial essential oils can be distilled from the leaves of this species, depending on where the trees occur (Bakkali et al. 2008).

In-depth investigation of this extract was carried out using various chromatographic techniques including thin layer chromatography (TLC), two-dimensional paper chromatography (TDPC), and column chromatography (CC). This work resulted in the isolation of 16 metabolites, including a novel compound, namely, myricetin  $3-O-\beta-^4C_1$ -galactopyranuronoid, together with 15 known metabolites which embrace a *C*-glycosyl chromone, nine flavonoids, and five phenols. All metabolites were characterized from this plant for the first time. Structures were elucidated by direct interpretation

of their spectral data, using HRESIMS, proton (<sup>1</sup>H), attached proton test (APT), hetero-nuclear single quantum coherence (HSQC), and hetero-nuclear multi bond connectivity (HMBC) NMR. As far as the available current literature is concerned there are no previous reports about the phenolics of that plant. In addition, in view of the published reports that phenolics, including flavonoids are major constituents of *Melaleuca* species (Hussein et al. 2007; El Toumy et al. 2001) and of the findings that hydroxy flavonoids are strong antioxidants, thus inducing appreciable immunological activity (Shrearma et al. 1996), we evaluated some immunological and biological effects of the extract in combination with a parallel cytotoxicity evaluation, using the MTT technique. Also, we assessed the antioxidant effectiveness, using the (DPPH) and the (ORAC) methods and evaluate the cytotoxicity for the extract and the new isolate against liver (Huh-7), breast (MCF-7) and prostate (PC-3) cancers using the neutral red assay (NRU) technique.

# 2. Investigations, results and discussion

Specimens of the dried leaves of *Melaleuca leucadendra* plant were exhaustively extracted with aqueous ethanol (3:1). The received extract was subjected to a series of column and preparative paper chromatographic (prep. PC) separations to isolate compounds **1-16**. Compound **14** has not been described before.

# 2.1. Structure elucidation

Compound **14** was isolated as a faint yellow amorphous powder. Its chromatographic properties, color reactions (dark purple on paper chromatogram (PC) under UV light turning orange with ammonia vapor and red color with the reduction Mg-conc. HCl test), together with the UV spectral data (see experimental) were consistent with those of flavonol glycosides.

Compound 14 exhibited a molecular mass ion at  $[M - 1]^{-}$ : m/z = 493.2628

Corresponding to a molecular mass of 494 and a molecular formula of  $C_{21}$  H<sub>17</sub> O<sub>14</sub>, as has been established by HRESIMS (calc.: 493.3513) in negative mode. After normal acid hydrolysis, compound **14** yielded galacturonic acid, and myricetin (comparative PC, COPC) and on controlled acid hydrolysis it yielded no intermediates, but gave finally the aglycone myricetin. Compound **14** is therefore, most probably, myricetin 3-*O*-galacturonoide.The suggested structure of

14 was confirmed by nuclear magnetic resonance (NMR) spectroscopy. From the <sup>13</sup>C NMR spectra, the presence of a galacturonic acid moiety followed from a resonance in the anomeric sugar region. The position of this resonance downfield at  $\delta$  102.64 ppm in comparison with the chemical shift (93.94 ppm) of the  $\beta$ -anomeric carbon in free pyranosidic galacturonic acid (Ramos et al. 1996) indicated that the galacturonic acid was attached directly from its anomeric hydroxyl to myricetin through an acetal bridge. That the galacturonic acid moiety must be attached to carbon number 3 of myricetin followed from the upfield shift of the resonance of this carbon and the accompanying downfield shift of the corresponding ortho and para-carbon resonances (in comparison with the corresponding resonances in free myricetin, see experimental). Similar shifts are well-known from the work of Markham et al. (1987). The  $\beta$  configuration of the galacturonic acid moiety was derived from its C-l chemical shift at  $\delta$  102.64 ppm (Garcia-Granados et al. 1980). The chemical shift values of all the galacturonic acid carbons confirmed the pyranose form of this moiety (Garcia-Granados et al. 1980).

The <sup>1</sup>H NMR spectrum of 14 was also in accordance with the proposed structure. In this spectrum the pattern of proton resonances (see experimental) revealed a resonance belonging to a substituted  $\beta$ -galacturonic acid anomer, whereby a distinct anomeric proton resonance was found located at  $\delta$  5.18 ppm with coupling constant = 8 Hz. The conformation of the galacturonic acid moiety is  ${}^{4}C_{1}$  as follows from the  $\beta$ -configuration discussed above. Confirmation of the final structure of 14 was achieved through extensive NMR analysis of its APT, HSQC and HMBC spectra, which allowed full assignment of all carbon and proton resonances. Theses spectral data (Table 1) unambiguously identified 14 as a myricetin  $3-O-\beta-4C_1$ - galactupyranouronoide whose flavonol carbon number 3 was located at  $\delta$  134.73 ppm. This upfield location (in comparison with the corresponding resonances in the spectrum of free myricetin (136 ppm) is obviously due to glycosylation at this carbon. Compound 14 was finally identified to be myricetin 3-O-  $\beta$ -<sup>4</sup>C,-galactopyranouronoide, a natural product which has not reported before in nature. It should be mentioned, however, that the recognizable biological activity of 14 necessitated the full identification of the configurational and conformational form of its galacturonoide moiety. The same structure with a galacturonoide moiety but different stereo structures could possess different biological activity.



Myricetin 3-O- $\beta$ - $^4C_1$ -galactopyranuronoid (14)

# 2.2. Known compounds

Noreungenin 8-C-glucosyl (1), gallic acid (2), catechin (3), caffeic acid (4), myricetin 3-O-rhamnoside (5), methyl gallate (6), ellagic acid 3- monomethylether-4-O-glucoside (7), quercetin 3-O-glucoside (8), kaempferol-3-O-glucoside (9), myricetin 3-O-glucoside (10), quercetin 3-O-rhamnoside (11), quercetin 3-rutinoside (12), myricetin 3-rutinoside (13), myricetin (15), quercetin (16).

# 2.3. Biological activities

## 2.3.1. Antioxidant activity

The radical scavenging activity of the crude extract and the new isolate **14** was first determined by the DPPH assay. The EC<sub>50</sub> values were 7.32 $\pm$ 2.71 µg/ml and 5.21 $\pm$ 0.87 µg/ml, respectively, compared to the positive control ascorbic acid with 1.8 $\pm$ 1.41 µg/ml.

Because of this high activity the anti-oxidative capacity was further investigated by the ORAC assay for the extract and the new isolate **14**. It demonstrated an  $EC_{50}$  value of  $8.25\pm3.22 \mu g/ml$  for

Table 1: NMR spectral data of 14 in DMSO-d<sub>6</sub> (400MHz for <sup>1</sup>H and 100.613 MHz for <sup>13</sup>C).

	$\delta_{_{\rm H}}(J,Hz)$	APT, $\delta_c$ (pp m)	HMBC correlations <sup>1</sup> H– <sup>13</sup> C
14			
Myricetin	n		
2		158.17 (quaternary)	
3		134.73 (methine)	
4		177.99 (quaternary)	
5		161.38 (quaternary)	
6	6.23 (broad s, $\Delta v_{12}$ =4Hz)	99.48 (methine)	
7	1/2	165.36 (quaternary)	H-6 to C-10, C-8
8	6.43 (broad s, $\Delta v_{12}$ =4Hz)	94.16 (methane)	
9	1/2	156.89 (quaternary)	H-8 to C-10, C-6
10		104.03 (quaternary)	
1'		119.63 (quaternary)	
2'	7.45(s)	109.40 (methine)	
3'		146.01 (quaternary)	H-2' to C-2, C-C-4'
4'		137.15 (quaternary)	
5'		146.01 (quaternary)	
6'	7.45(s)	109.42 (quaternary)	
Galactu-			H-6' to C-2, C-C-4'
ronic			
1"	5.18 ( <i>d</i> , <i>J</i> =8 Hz)		
2"	3–4.1 ( <i>m</i> , galacturonic & H <sub>2</sub> O protons)	103.68 (methine)	
3"	3–4.1 ( <i>m</i> , galacturonic & H <sub>2</sub> O protons)	72.12 (methine)	H-1" to C-3
4"	$3-4.1$ ( <i>m</i> , galacturonic & $H_2O$ protons)	74.05 (methine)	
5"	3–4.1 ( <i>m</i> , galacturonic & H <sub>2</sub> O protons)	70.72 (methine)	
6"	3–4.1 ( <i>m</i> , galacturonic & H <sub>2</sub> O protons	75.26 (methine)	
	2 -	61.63 (methylene)	

the crude extract and higher activities of the isolated compound **14**,  $4.56\pm0.82 \ \mu$ g/ml. These values are lower than that of the positive control Trolox which had an ED<sub>50</sub> of 27.0±13.41  $\mu$ g/ml.

## 2.3.2. Cytotoxicity against tumor cell lines, NRU assay

NRU assay was used to assess the cytotoxicity of the *Melaleuca* extract and the new isolate **14** against three different solid tumor cell lines. The IC<sub>50</sub> values for cytotoxicity of the extract and the isolated compound **14** are given in Table 2. The vehicle in which the test samples were dissolved had no influence on measured parameters. Using etoposide (positive control for cytotoxicity) viability of the three tested cell lines was reduced to 40 to 60%. The extract and the novel compound showed the highest cytotoxic activity against colon cancer cell line HCT 116 with IC<sub>50</sub> of 83.08±0.71µg/mL and 24.08±1.57 µg/mL respectively.

Table 2: Cytotoxicity  $(IC_{s_0} \mu g/m)$  of the extract of Melaleuca leucadendra and the new isolate 14

Liver carcinoma cell line (Huh-7)	Breast cancer MCF-7	Colon cancer HCT 116	
$104 \pm 3.55$	$102.61 \pm 5.91$	$83.08 \pm 0.71$	Extract
$35.76 \pm 2.51$	$31.08 \pm 3.83$	24.08 ± 1.57	Myricetin 3- <i>O</i> β- <sup>4</sup> C <sub>1</sub> -ga- lacturonide

## 2.3.3. Determination of phagocytic activity

Heparinized (10 IU/ml) rat blood grouped samples were centrifuged at 3000 rpm for 10 min, then the buffy coat was aspirated and carefully layered on histopaque neutrophil isolation medium (Biowest), pH 7.3 by a ratio 1:3 in a siliconized centrifuge tube and the gradient was centrifuged at 990 rpm for 25 min at 4 °C. The pellet containing neutrophils and RBCs was transferred into 50 ml sterile falcon tube. A total of 20 ml sterile, cold distilled water was added and let stand for, exactly 45 s to lyse RBCs. Isotonicity was restored by the addition of 10 ml of sterilized normal saline, and then the content was centrifuged at 3000 rpm for 10 min. The pellet was washed 3 times by Hanks balanced salt solution (HBSS) and re-suspended in 1 ml and kept at 4 °C until used (Hogan et al. 1990).

Twenty four hours tryptic soy broth culture of *Staphylococcus aureus* was adjusted to 52.5% transmission as each 1 ml contained  $5x10^7$  to  $10^8$  CFU. The broth containing the bacteria was centrifuged at 3000 rpm for 10 min and the pellet was washed twice with HBSS. The bacteria were opsonized with 1 ml of 10% homologous serum (collected from 5 different rat sera) for 30 min at 37 °C with gentle shaking. After opsonization, the bacteria were centrifuged, washed once and suspended in 1ml HBSS (Silva et al. 1988).

Ten-microliter volumes of bacterial suspensions were added to neutrophil phagocytic cells in a 10:1 ratio obtained from different concentration plant extract groups in 1 ml of HBSS and incubated for 2 h at 37 °C under rotation. Subsequently, the cells were centrifuged for 4 min at 110 x g and the supernatant was removed. The pellet was streaked on clean glass slides, and stained by the addition of 200  $\mu$ l of acridine orange (15 mg/liter) for 1 min. The slide preparations were examined under Axio Imager Z2 fluorescence microscope using transmitted light. Photographs were taken using AxioCam MRc3 S/N 4299 film with the shutter set for 2 min (Nagl et al. 2002). These results proved that *Melaleuca leucadendra* extract led to a significant increase in phagocytic index of neutrophils by increasing the dose. These findings suggest an immunostimulant behavior.

# 3. Experimental

#### 3.1. General

NMR spectra were acquired in DMSO-d<sub>6</sub> on a Bruker Avance 400 NMR spectrometer, at 400 MHz. Standard pulse sequence and parameters were used to obtain 1D- <sup>1</sup>H and <sup>13</sup>C APT, and 2D- HSQC and HMBC spectra. <sup>1</sup>H chemical shifts (\delta) were measured in ppm, relative to TMS and <sup>13</sup>C NMR chemical shifts to DMSO-d<sub>6</sub> and were converted to TMS scale by adding 39.49. 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 Biosystems, USA). 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. ORAC measurements were performed on a FLUOstar Omega Microplate Reader - BMG LABTECH. UVrecording was made on a Shimadzu UV–Visible-1601 spectrophotometer and optical rotation was obtained on a Kruess optronic Polarimeter. Paper chromatographic analysis (PC) was carried out on Whatman No. 1 paper, using solvent systems: (1)  $\rm H_2O$ ; (2) 6% HOAc; (3) BAW (n-BuOH–HOAc–H\_2O, 4:1:5, upper layer)

## 3.2. Plant material

Leaves of *Melaleuca leucadendra* were collected from the Zoo garden at Cairo, on September, 2016 and identified by Prof. Salwa Kawashty at the Department of Phytochemistry and Plant Systematic, National Research Centre (NRC), Cairo, Egypt. A voucher specimen (M 1732) has been deposited at the herbarium of the NRC.

## 3.3. Preparation of extract

Fresh leaf material (2 kg) was extracted with hot EtOH/H<sub>2</sub>O (3:1, 3 times, each with 3 l, for 8 h, under reflux). The solvent was evaporated under vacuum. The resulting dry sticky material thus left (200 g), dissolved in 200 ml H<sub>2</sub>O, was applied to a Polyamide 6S chromatographic column (Riedel-de Haen, Seelze, Hannover, Germany) and eluted with H<sub>2</sub>O, followed by H<sub>2</sub>O/MeOH mixtures of decreasing polarity to yield ten major fractions (I – X). Following removal of the solvents the received dried ten fractions were as follow: fraction I eluted with H<sub>2</sub>O; II with 10 %, III with 20 %, IV with 30 %, V with 40 %, VII with 50 %, VII with 60 %, VIII with 70 %, IX with 80 % and X with MeOH). The fractions individually collected were subjected to two dimensional paper chromatography (TDPC).

# 3.4. Isolation and identification of phenolics

Compound 1 (37 mg) was purely isolated from fraction II (2.5 g, eluted with H<sub>2</sub>O) by repeated column fractionation over MCI-gel, using H<sub>2</sub>O for elution followed by prep. PC for subfraction 3, using BAW (n-BuOH-HOAc-H2O, 4:1:5, upper layer) as solvent. Compound 2 (108 mg) was separated pure from fraction 3 (2.6 g eluted with 20 % aqueous MeOH) by Sephadex LH-20 column fractionation, using n-BuOH water saturated with  $H_2O$  for elution, followed by crystallization from  $H_2O$ . Compound **3** was isolated from fraction IV (8.17 g, eluted with 40 % aqueous MeOH) by MCI gel column fractionation, using H<sub>2</sub>O as solvent, followed Prep. PC, using BAW as solvent. Compound 4 (29 mg) was purely isolated from 1.3 g of fraction V (eluted by 40 % aqueous MeOH) by extraction by EtOAc, the residue dissolved in ethanol, filtered and the dried filtrate was applied on MCI gel column eluted with 50 % aqueous MeOH and the eluate was then purified over Sephadex LH-20 using 50 % aqueous MeOH as solvent to afforded pure sample of compound 5 (24 mg). Fraction VI (1.56 g, eluted with 60% aqueous MeOH) was fraction-ated into  $H_2O$  and MeOH sub-fractions by being applied to MCI gel column. The MeOH sub-fraction was dried and extracted with ether. The ether contained pure compound 6 (23 mg). The residue was subjected to prep. PC using 6 % aqueous acetic acid as solvent, whereby, compounds 7 (18 mg), 8 (21 mg), 9 (17 mg), 10 (30 mg) were individually separated. Fraction 7 (1.56 g, eluted with 70 % aqueous MeOH) was applied to a Sephadex LH-20 column, eluted with n-BuOH saturated with H<sub>2</sub>O and the eluted dark purple band (under UV) was dried under vacuum, Prep. PC of the received dried material using 6 % aqueous acetic acid afforded a pure sample of compound 11 (19 mg). Compound 12 (20 mg) was isolated pure from fraction 8 (1.5 g, eluted with 80 % aqueous MeOH) by prep. PC and elution with 6 % ageous acetic acid. Prep. PC of fraction IX (1.8 g, eluted with 90 % aqueous MeOH) using 30 % aqueous acetic acid as solvent yielded another amount of pure sample of compound **12** (34 mg) beside pure sample of **13** (22 mg). Fraction X (1.5 g, eluted with MeOH) was exhaustively extracted with ether, the ether was filtered on and removed under vacuum. The ether insoluble portion was subjected to repeated prep. PC (3 times), using 6 % aq, acetic acid to yield a chromatographically pure sample of 14 (30 mg). Application of the received ether extract on a silica gel column and elution with n-hexane:EtOAC (1:1) afforded pure samples of 15 (16 mg) and 16 (12 mg). *Myricetin 3-O-* $\beta$ - $^4C_1$ -galactopyranuronoide (14): A faint yellow amorphous powder; [ $\alpha$ ]

hyperin Jorder (19), Cr genetopytaminotae (14), Think yelow antophoto, power, BAW). UV  $\lambda_{max}$  nm in MeOH: 266, 267, 295, 36; NaOAc: 235, 274; NaOAc-H<sub>3</sub>BO<sub>3</sub>: 260, 302, 386; AlCl<sub>3</sub>: 270, 303, 393; Complete acid hydrolysis of **14** (11 mg in 5 ml, 2 N aq. HCl, at 100 °C for 2 h) yielded myricetin and galacturonic acid; Controlled acid hydrolysis (8 mg, 10% aq. AcOH, 30 min, 100 °C) yielded only myricetin at the end; HRESI-FTMS (negative ions) of **14**: [M – 1]<sup>-</sup> : m/z = 493.2628 corresponding to a molecular mass of 494 and a molecular formula of C<sub>21</sub> H<sub>17</sub> O<sub>14</sub> as has been established by HRESIMS (calc.: 493.3513) in negative mode. 1-D and 2-D <sup>1</sup>H and <sup>13</sup>C NMR data: Table 1.

# 3.5. Biological assays

## 3.5.1. Radical scavenging effect, DPPH assay

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

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carried out in triplicate. Ascorbic acid was used as positive control. Percentage radical scavenging activity of samples was calculated using the following formula:

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

ED50 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.

## 3.5.2. Oxygen radical absorbance capacity, ORAC assay

Reactive oxygen species, ROS are generated by the thermal degradation of AAPH and quench the signal of the fluorescent probe fluorescein. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of the fluorescheme. A vitamin E derivate, 6-hydroxy-2,5,7,8-tetra-methylchroman 2-carboxylic acid (Trolox), was used as positive control. Test compound **14** was dissolved in phosphate buffered saline (10 mM, pH 7.4) and investigated for its antioxidant capacity. Experiments were done in black 96-well plates. In each well of a 96-well plate 150 µf llourescein (final concentration: 2.5 nM), 25 µf Trolox (final concentrations: 0.78 – 25 µM) or 25 1 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 25µl 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 (Mishra et al. 2012).

#### 3.5.3. Cytotoxicity assay

Human hepatocellular carcinoma cell line (Huh-7), colorectal adenocarcinoma cell line (HCT-116) and breast adenocarcinoma cell line (MCF-7) were obtained from the Vaccera (Giza, Egypt). Cells were cultured in RPMI 1640 medium (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 8 % fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) at 95% humidity, 5% CO<sub>2</sub> and 37 °C. HaCaT cells were subcultured twice a week and regularly tested for mycoplasma. Cytotox-icity of test samples against the three cell lines was investigated using the neutral red uptake (NRU) assay. After 24 h cultivation in 96 well plates (3 or 8 x 10<sup>3</sup> cells/ well) medium was removed and cells were exposed for 72 h to various concentrations (max. 500 µg/ml) of test samples. After removal of the medium wells were washed with HBSS (Hanks Balanced Salt Solution, PAA). Cells were than incubated for 3 h with 100 µl 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red, NRU,

Merck, Darmstadt, Germany, stock solution 3.3 µg/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 IC<sub>50</sub> values were defined from obtained dose-response curves and expressed in mean±SD. All compounds were tested in duplicate. Etoposide (Alexis Biochemicals,  $\geq$  98 % purity) was used as positive control (Amarowicz and Pegg 2013).

Conflicts of interest: None declared.

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