Cytoprotective Effects of 5 Benzophenones and a Xanthone from *Hypericum annulatum* in Models of Epirubicin-Induced Cytotoxicity: SAR-Analysis and Mechanistic Investigations

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Abstract: A new benzophenone *O*-glucoside neoannulatophenonoside (**1**) together with the known pinocembrin-7-*O*-glucoside were isolated from the aerial parts of *Hyperium annulatum* Moris (*Guttiferae*). The former was identified as 3',5',6-trihydroxy-4-methoxybenzophenone-2-*O*-*β*-D-glucopyranoside by means of chemical and physical evidence. The cytoprotective effects of the new compound together with the previously isolated from this species hypericophenonoside (**2**), annulatophenone (**3**), annulatophenonoside (**4**), acetylannulatophenonoside (**5**) and 1,3,7-trihydroxyxanthone (**6**) were evaluated in a model of epirubicin-induced cellular toxicity in K-562 cells. While the benzophenone *O*-glycosides **1**, **2**, **4** and **5** exerted substantial cytoprotective effects against the epirubicin cytotoxicity in K-562 cells the aglycones **3** and **6** lacked any significant cytoprotective activity. Biochemical investigations aimed at evaluating the free-radical scavenging activity of the tested compounds as well as their effects on the cellular glutathione stores were carried out as well, aiming at unravelling the mechanisms of cytoprotection. Finally, the ability of **1**, **4** and **5** to ameliorate epirubicin-induced anticlonogenic effects on bone marrow cells colony forming units, *in vitro* were also evaluated. Taken together, the experimental data indicate that the benzophenone glycosides isolated from *H. annulatum* have a substantial cytoprotective potential against the toxic effects induced by epirubicin and necessitates further detailed pharmacological evaluation of these compounds as possible chemoprotective/radioprotective agents.

Key Words: *Hypericum annulatum*, Guttiferae, benzophenones, neoannulatophenonoside, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, GSH, benzophenones, cytoprotection, epirubicin.

INTRODUCTION

The contemporary modalities for treating cancer – radiation therapy and chemotherapy are significantly limited in their ability to distinguish between normal and malignant cells. Thus cancer therapy is typically associated with severe adverse reactions upon rapidly proliferating cellular populations such as bone marrow and epithelial cells in the mouth, digestive tract, reproductive system, and hair follicles [1,2]. This unfavorable toxicological profile generally limits the possibility to gain benefit by increasing the dose intensity. Furthermore, cancer treatment modalities could also substantially decrease the quality of life, and pose life-threatening risks to patients. An innovative strategy towards amelioration of the detrimental effects, associated with high dose chemo- or radiotherapy has been to search for cytoprotective agents, which preferentially protect normal tissue cells from damage resulting from radiation therapy or radio-chemotherapy [3].

Considering the fact that oxidative stress triggered by rise in free radical formation and/or decreased antioxidant defence capacity is strongly implicated in the toxicity of chemo- and radiation therapies much effort has been focused upon the research of diverse antioxidants as potential chemo- or radioprotective agents [4-6]. Among these, a variety of natural polyphenols, e.g. quercetin and silymarin, have shown to modulate the toxic effects of chemotherapeutic agents and have been considered as possible chemoprotective agents for clinical use [7,8].

Besides the extensively investigated flavonoids, other classes of plant polyphenols that also possess structural prerequisites for antioxidant activity include different phloroglucinol derivatives, xanthones and substituted benzophenones, characteristic for the species of the Guttiferae family [9,10].

Hypericum (Guttiferae) is a large genus of herbs or shrubs, which grows widely at temperate regions [11], and is used as traditional medicinal plants in various parts of the world [12]. Phytochemical investigations of the species belonging to *Hypericum* genus led to isolation mainly of flavonoids [13], xanthones [14, 15], naphthodianthrones [16] and phloroglucinols [17]. *Hypericum annulatum* Moris subsp. *annulatum* also known as *H. degenii* Bornm. is a herbaceous plant, growing at the Balkan Peninsula and Sardinia [18,19]. In previous studies of this plant the presence of flavonoids, catechins, hypericins, xanthones,

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benzophenones and a prenylated phloroglucinol was demonstrated [20-24].

In continuation of our work on *H. annulatum* we now report the isolation and characterization of a new benzophenone *O*-glucoside. The cytoprotective potential of this compound together with the previously isolated from this plant benzophenones annulatophenone, annulatophenonoside, acetylannulatophenonoside, hypericophenonoside and the xanthone genistein (1,3,7-trihydroxyxanthone) utilizing *in vitro* models of epirubicin-induced cell-injury was tested. In addition, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the compounds was also investigated. A biochemical assay for glutathione (GSH) levels was carried out in order to elucidate the mechanism underlying the cytoprotective potential of the tested compounds. Finally, the ability of some of the benzophenone *O*-glycosides to ameliorate epirubicin-induced anticlonogenic effects on bone marrow cells colony forming units *in vitro* was also evaluated.

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points (m.p.) were measurer on a Kofler hot-stage microscope and are uncorrected. Optical rotations (OR) were obtained using Perkin-Elmer 241 polarimeter. Ultraviolet (UV) spectra were run in MeOH on a Specord UV-VIS instrument. Infra red (IR) spectra were recorded with Shimadzu FTIR-8101 M. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker ARX 300 apparatus at 300 and 75 MHz, respectively, in DMSO-d_6, using tetramethylsilane (TMS) as internal standard. The solvents were of HPLC grade and purchased from Merck (Germany). Electrospray (ESI) mass spectra were recorded with a Bruker Esqire ~LC mass spectrometer by flow injection using a methanol/water mixture (50/50, v/v) with 1% acetic acid.

Plant Material

The aerial parts of *Hypericum annulatum* Moris (syn. *H. degenii* Bornm.) (Guttiferae) were collected during the flowering season from a wild habitat at the Central Rhodope Mountains in July 1997. A voucher specimen (No. 144296) was deposited at the Herbarium of Botany Institute of Sofia (SOM).

Extraction and Isolation

The air dried and powdered herb (1.7 kg) was refluxed with *n*-hexane (6×1 L) at 60 ºC for 2 h each time and then extracted with hot MeOH (8×6 L) at 70 ºC. The crude MeOH residue was dissolved in hot H_2O (2 L), filtered and treated with $CHCl_3$ (10×300 mL). The aq. phase was extracted with EtOAc (7×500 mL). The combined EtOAc layers were concentrated with a rotary evaporator to remove the solvent. Aqueous layer was then extracted with H_2O saturated *n*-BuOH (10×300 ml). The *n*-BuOH part was evaporated to dryness and the residue (130 g) was treated with Me_2CO under reflux (10×300 ml) at 50ºC. The Me_2CO solution was filtered and evaporated to dryness to give dark brown residue (86 g), which was subjected to column chromatography (CC) on polyamide S 6 (particle size < 160 μm, 258 g, Riedel-de Haën, Germany). The column was eluted using a stepwise gradient water and water-EtOH mixtures (water, 10 % EtOH and 10→50 % EtOH in steps of 5 %). A total of 43 fractions (500 mL each) were collected. Fractions thus collected were analyzed through TLC on silica gel 60 F_{254} aluminium sheets (Merck, Germany) using $CHCl_3$-MeOH-H_2O (70:20:3 and 70:30:3) as mobile phases separately. Spots were detected under UV (254 and 366 nm). A 0.5 % solution of Fast Blue B Salt (Fluka, Germany) in MeOH-H_2O (1:1) was used as spraying agent. Based on above TLC techniques the fractions were combined to give four pooled fractions (A-D): 1-2 = fr A (eluted with water), 3-23 = B (eluted with water), 24-37 = C (eluted with water and 10-35 % EtOH) and 38-43 = D (eluted with 40-50 % EtOH). Fraction B (26.4 g) was chromatographed on silica gel (70-230 mesh, 368 g, Merck, Germany) CC, eluted with $CHCl_3$-1% AcOH in MeOH mixtures (90:10→81:19, in steps of 1, 1000 mL of each mixture). A total of 49 fractions (200 mL each) were collected. These were combined according to TLC analysis to give 10 pooled fractions (B_1-B_{10}): 1-4 = fr B_1 (90:10), 5-7 = fr B_2 (90:10, 89:11), 8-10 = fr B_3 (89:11), 11-14 = fr B_4 (88:12), 15-23 = fr B_5 (88:12→86:14), 24-30 fr B_6 (86:14, 85:15), 31-33 fr B_7 (84:16), 34-40 = fr B_8 (84:16→82:18), 41-47 = fr B_9 (82:18, 81:19) and 48-49 = fr B_{10} (81:19). Fraction B_5 (1.64 g) was chromatographed on Sephadex LH-20 column (45 cm × 3 cm, Pharmacia, Sweden) and MeOH as eluent. A total of 10 fractions (30 mL each) were collected. The fraction 4 (227 mg) was subjected to preparative TLC on silica gel 60 plates (layer thickness 0.5 mm, 20_20 cm, Merck, Germany) using $CHCl_3$-MeOH-AcOH-H_2O (76:20:1:3) as mobile phase. About 10 mg of the fraction were applied on a single plate. Gel filtration over Sephadex LH-20 (eluent MeOH) was used as final purification step, which gave 56 mg of compound **1**. The chromatographic separation of the EtOAc residue (57 g) and characterization of isolated compounds, namely 2,4,5',6-tetrahydroxybenzophenone-2'-*O*-β-D-glucopyranoside (hypericophenonoside) (**2**), 2,3',5',6-tetrahydroxy-4-methoxybenzophenone (annulatophenone) (**3**), 3',5',6-trihydroxy-4-methoxybenzophenone-2-*O*-α-L-arabinofuranoside (annulatophenonoside) (**4**) and 3',5',6-trihydroxy-4-methoxybenzophenone-2-*O*-α-L-3"-acetylarabinofuranoside (acetylannulatophenonoside) (**5**) have been described elsewhere [22,23]. In addition, from EtOAc fraction pinocembrin-7-O-glucoside was also isolated by usual techniques and was identified by spectral methods (UV, IR, MS, NMR), co-chromatography with authentic samples and melting point.

Acid Hydrolysis of Compound 1

A solution of compound **1** (10.0 mg) in 1N HCl (2 mL) was stirred at 100 °C under reflux in a reaction flask for 4 h. Reaction mixture was transferred to separating funnel, diluted to 10 mL with water and extracted with water saturated EtOAc (3 × 5 mL). Both layers were evaporated to dryness in vacuo separately. The EtOAc residue was subjected to gel filtration on Sephadex LH-20 (20 cm × 1.5 cm) and eluted with MeOH. The aglycone of **1** was obtained as pale yellow powder (5 mg) and was identified as 2,3',5',6-tetrahydroxy-4-methoxybenzophenone **3** on the basis of melting point, co-TLC [silica gel 60 F_{254}; mobile phases:

$CHCl_3$-Me_2CO-HCOOH (76:16:4), $CHCl_3$-MeOH (9:1), benzene-EtOAc-MeOH (4:4:1); detection: under UV (254 nm) and spraying with solution of Fast Blue B Salt] with a reference compound, UV, IR and ESI-MS [22]. The sugar was identified as D-glucose by means of co-TLC [cellulose (Merck, Germany), mobile phase EtOAc-Pyridine-H_2O (12:5:4), anisidine phthalate reagent and heating to 110 ºC for 3-5 min] with reference compounds.

Isolation of Compound 6

A solution of hypericophenonoside **2** (100 mg) was dissolved in 1N HCl (5 mL) and was stirred at 100 °C under reflux in a reaction flask for 4 h. Thus produced yellow precipitate of compound **6** was filtered, washed with water and recrystallized from MeOH (48 mg).

Isolates

3',5',6-trihydroxy-4-methoxybenzophenone-2-O-β-D-gluco-pyranoside (neoannulatophenonoside) (1)

Pale brown amorphous powder; m.p.: 137-138 ºC; $[\alpha]_D^{\sim}$ -26.06 (*c* 1.005, MeOH); UV (MeOH): λ_{max} (log ε) = 281 (4.17), 297sh (3.95); IR (nujol): ν = 3000-3500 (broad), 1651, 1615, 1556 cm^{-1}; ^{1}H-NMR (DMSO-d_6 ;300 MHz) δ = 6.58 (d, *J* = 2.2 Hz, H-2', H-6'), 6.38 (t, *J* = 2.2 Hz, H-4'), 6.22 (d, *J* = 2.0 Hz, H-3), 6.16 (d, *J* = 2.0 Hz, H-5), 4.76 (d, J = 7.8 Hz, H-1"), 3.70 (s, MeO), 3.39-3.45 (m, H-6"), 3.20-3.29 (m, H-3", H-5"), 2.94-3.08 (m, H-2", H-4"), hydroxyl protons are exchanged; ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 194.71 (C=O), 110.83 (C-1), 156.36 (C-2), 92.16 (C-3), 161.06 (C-4), 95.28 (C-5), 158.43 (C-6), 140.29 (C-1'), 107.04 (C-2'), 158.43 (C-3'), 107.04 (C-4'), 158.43 (C-5'), 107.04 (C-6'), 100.75 (C-1"), 73.22 (C-2"), 76.66 (C-3"), 69.72 (C-4"), 77.33 (C-5"), 60.75 (C-6"); Positive ESI-MS: *m/z* (%) = 461 $[M + Na]^+$ (100), 439 $[M + 1]^+$ (11), 277 $[M_{agl} + 1]^+$ (29), 167 $[C_8H_7O_4]^+$ (12), 137 $[C_7H_5O_3]^+$ (3).

1,3,7-trihydroxyxanthone (genistein) (6)

Yellow needles: HREI-MS: m/z (rel. int.) = 244.0383 (M^+, 100); m. p., IR, UV and ^{1}H-NMR data are the same as in [20].

Cell Lines, Culture Conditions and Treatment

The myeloid K-562 cells were supplied from the German Collection of Microorganisms and Cell Cultures. The cells were maintained in a controlled environment (RPMI-1640 medium, supplemented with 10 % heat-inactivated fetal calf serum and 2 mM L-glutamine, at 37°C in a 'Heraeus' incubator with 5% CO_2 humidified atmosphere). In order to maintain the cells in log phase cellular suspension aliquots were discarded and the cells were re-fed with fresh RPMI-1640 medium two or three times/week. The stock solutions of the tested compounds were freshly prepared in water and consequently diluted in RPMI-1640. For each concentration at least 6 wells were used.

Cytotoxicity Determination (MTT-Dye Reduction Assay)

The cytotoxicity screening was carried out in order to define non-cytotoxic concentrations for the cellular protection studies. We used the xanthone genistein **6** as positive control throughout the screening panel, since it was found to be a major product from the acid/enzymatic hydrolysis of compound **2** [22] and probably represents a major biotransformation product following oral intake of **2**. The 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Sigma) dye reduction assay was carried out as described by Mossmann [25] with some modifications [26]. Briefly, 100 μL aliquots of cell suspension (1×10^5 cells/mL) were seeded in 96-well microplates. Following a 24 h incubation at 37°C the cells were exposed to the tested compounds for 72 h. After the exposure period 10 μL MTT solution (10 mg/mL in PBS) aliquots were added to each well and the plates were further incubated for 4 h at 37°C. The MTT-formazan crystals formed were dissolved by addition of 100 μL/well 5% formic acid in 2-propanol (Merck) and the absorption of the samples was measured with an ELISA reader (Uniscan Titertec) at 580 nm. The results were expressed as survival fractions (% of untreated control). From the experimental data the corresponding concentration-response curves were constructed and the IC_{50} values, i.e. the concentrations causing half-maximal suppression of cell viability were calculated. In order to allow a quantitative merit for the cytoprotective effects of the tested agents the IC_{50} values obtained after their combined application with epirubicin were divided by the IC_{50} value of epirubicin administered alone. The corresponding ratios are herein referred as protection indices.

DPPH Radical-Scavenging Activity

Experiments were carried out according to the method of [27] with a slight modification [12]. Briefly, a 0.1 mM solution of DPPH (Fluka, Germany) in MeOH and 1 mM solutions of investigated compounds were prepared. Then, 2 ml of DPPH solution was mixed with 2 ml of sample solution. Finally, after 30 min, the absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated in the equation:

$$\%DPPHradical-scavenging = \frac{A_{control} - A_{sample}}{A_{control}} 100 ,$$

where $A_{control}$ is the absorbance of the control solution, while A_{sample} is the absorbance of the sample solution.

The DPPH solution without sample was used as control. In the measurements of the activities, two independent experiments were made, and results were calculated as means of these measurements. To determine whether there was any differences between activities of samples, analysis of variance was also applied to the results. Values of $p < 0.05$ were considered as significantly different ($\alpha = 0.05$). Ascorbic acid and butylated hydroxyanisole (BHA) (Fluka, Germany) were used as positive controls.

Evaluation of Glutathione Levels

For the determination of the GSH levels a spectrophotometric assay was used as described previously [28]. In

brief 2-5×10^5 K-562 cells per sample were washed in 1 mL PBS and centrifuged at 6000 rpm for 5 minutes. The cell pellet was then lysed in 100 μL 0.5% solution of Triton X-100 in 0.2 M EDTA at 4ºC for 5 minutes. Protein was thereafter precipitated through addition of 20 μL 20% (v/w) trichloroacetic acid. The volume of each sample was then adjusted to 200 μL with distilled water and following centrifugation of the samples at 145000 rpm for 10 minutes the supernatants were assayed for GSH content. Supernatant aliquots were transferred in 96- well microplates (100 μL per well) together with 160 μL Tris buffer (0.4M, pH 8.9) and 4 μL of 5,5-dithio-bis(2-nitrobenzoic acid) 3.4 mg/mL solution in MeOH. The yellow product yielded was measured at 414 nm using Multiscan, Titertek ELISA reader. GSH content was calculated by means of standard curve and expressed as a percentage of untreated control.

Long-Term Bone Marrow Cell Culture (LTBMCC) and CFU-GM

Murine LTBMCC were prepared as previously described [29]. In brief female mice C57BL6 (Slivnitza, Bulgaria) served as donors. After euthanizing the animals by cervical dislocation, their femurs were obtained and the bone marrow was flushed into LTBMCC medium (MEMα, supplemented with 10 % FCS, 10 % horse serum, 1 μM hydrocortisone, 0.017 mg/mL transferrin, 0.007 μL/mL β-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B). Bone marrow 1×10^7 cells were plated in 25 cm^2 cell culture flasks and incubated at 37ºC and 5% CO_2 humidified atmosphere. Weekly, half of the medium in each flask was removed and an aliquot of fresh medium was added. After an adherent feeder layer had been established, the cultures were refed with 1×10^7 bone marrow cells per flask. After incubation the cells were exposed to the benzophenones **1**, **4**, **5**, epirubicin or combinations thereof for 24 h. Thereafter, the cultivation medium was discarded, the cells were harvested *via* trypsinization, and 250 00 cells were plated in 5 mL of semi-solid medium (0.8 % RPMI-methylcellulose, 30 % horse serum and 0.1 ng/mL recombinant murine granulocyte/macrophage-colony stimulating factor). Aliquots of 1 mL per dish (4 dishes per treatment group) were seeded in Petry dishes and cultivated for 10 days. Colonies (clusters consisting of 20 or more cells) were counted using inverted microscope.

Statistics

The data processing included the Student`s t-test with $p \leq 0.05$ taken as significance level, using Microsoft EXCEL and OriginPlot software for PC.

RESULTS AND DISCUSSION

An extensive chromatographic procedure including column chromatography on polyamide and silica gel, gel filtration over Sephadex LH-20 and preparative TLC of the Me_2CO soluble part of the *n*-BuOH fraction of *H. annulatum* gave a pale yellow amorphous powder of compound **1**. It was easily dissolved in water and gave a dark brown colour with ferric chloride. The IR spectrum of **1** showed absorption bands at 3000-3500 (chelated OH); 1651 (C=O); 1615, 1556 (delocalized C=C) cm^{-1}. The bathochromic shift of the band at 340 nm in the UV spectrum with $AlCl_3$ (+59) revealed the presence of a free hydroxyl group in *ortho* position to the carbonyl function. The ESI-MS of compound **1** showed a molecular ion at m/z = 439 $[M+1]^+$, consistent with an elemental formula of $C_{20}H_{22}O_{11}$. Acid hydrolysis of **1** gave 2,3',5',6-tetrahydoxy-4-methoxybenzophenone (annulatophenone) and D-glucose. The ^{1}H-NMR spectrum of compound **1** showed five signals at δ 6.16 (d, *J* = 2.0 Hz) (ring A), 6.22 (d, *J* = 2.0 Hz) (ring A), 6.58 (d, *J* = 2.2 Hz) (ring B), 6.38 (t, *J* = 2.2 Hz) (ring B) and 3.70 (s) (MeO at C-4), which belong to aglycone moiety. This signal pattern was similar to that of annulatophenonoside (**4**) and acetylannulatophenonoside (**5**) [23]. The symmetry of ring A was broken by attaching of a glucosyl moiety to position 2 of annulatophenone (**3**), which was shown by two doublets at δ 6.22 (d, *J* = 2.0 Hz) and 6.16 (d, *J* = 2.0 Hz). This fact unambiguously points to C-2 as the linkage position of the glucose. The signals of the sugar protons appeared at δ 3.39-3.45 (m, H-6"), 3.20-3.29 (m, H-3", H-5") and 2.94-3.08 (m, H-2", H-4"). The doublet at δ 4.76 (d, J = 7.8 Hz) was attributed to anomeric proton in the glucose moiety and suggesting its β-configuration. The signals corresponding to the 6 sugar carbons at δ 100.75, 73.22, 76.66, 69.72, 77.33 and 60.75 are typical for glucopyranosides [30]. The assignment of the signals was confirmed by ^{1}H-^{1}H COSY-, HETCOR- and COLOC-experiments. Multiplicities of the ^{13}C-NMR signals were revealed by DEPT experiments. Finally, compound **1** was identified as 3',5',6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucopyranoside (Fig. (**1**)), named neoannulatophenonoside. This compound is a new natural product. In addition, from the EtOAc fraction of *H. annulatum* MeOH extract pinocembrin-7-O-glucoside was also isolated and its spectral data were in good agreement with that given in the literature [31]. Despite being a known compound, pinocembrin-7-O-glucoside is reported here for the first time for *Hypericum* species.

The sole administration of epirubicin caused a significant decrease of the cell viability of K-562, with practically total eradication of vital cells at concentrations above 2.5 μM. The experimentally obtained value of IC_{50} was 0.78 μM. The benzophenone derivatives as well as the xanthone were found to be practically non-toxic at concentrations up to 50 μM and conversely they were co-administrated with epirubicin at non-toxic concentrations (12.5 μM and 25 μM) in order to evaluate their effects upon the anthracycline-induced cytotoxicity.

As evident from the results, summarized in Table **1** and Fig. (**2**), compounds **1**, **2**, **4** and **5** exerted a prominent protection of the cellular population against the cytotoxic effects of the antineoplastic drug and caused a shift of the concentration response curve with subsequent elevation of the IC_{50} values and decrease of the maximal efficacy assessed at the higher concentration evaluated. The analysis of the obtained protection indices revealed that the cytoprotective potential was concentration-dependant, i.e. it was more pronounced at the higher concentration of the polyphenols applied. Both genistein **6**, and annulatophenone **3**, applied at non-cytotoxic concentrations lacked substantial cytoprotective activity in the model system used. This is

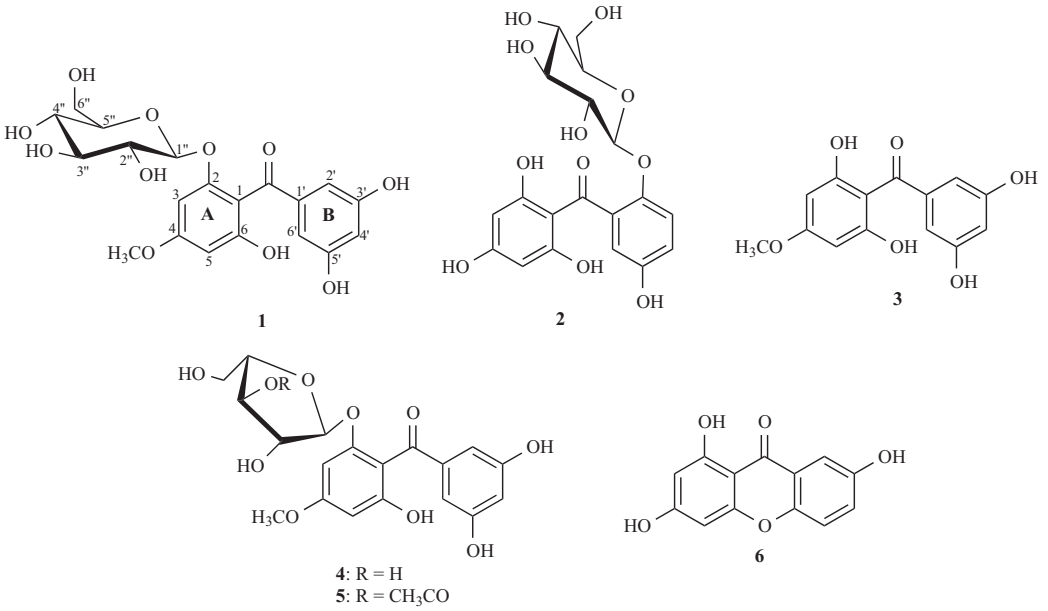

Fig. (1). Structures of the tested compounds.

evident from both the concentration response curves and the IC_{50} values obtained.

Table 1. Cytoprotective Activity of Compounds 1-6 Against Epirubicin-Induced Cytotoxicity in K-562

Treatment groups	IC_{50} value	PI[a]
Epirubicin alone	0.78 μM	-
Epirubicin + **1** (12.5 μM)	2.53 μM	3.24
Epirubicin + **1** (25 μM)	3.61 μM	4.62
Epirubicin + **2** (12.5 μM)	0.88 μM	1.13
Epirubicin + **2** (25 μM)	1.47 μM	1.88
Epirubicin + **3** (12.5 μM)	0.79 μM	1.01
Epirubicin + **3** (25 μM)	1.02 μM	1.29
Epirubicin + **4** (12.5 μM)	1.75 μM	2.24
Epirubicin + **4** (25 μM)	2.44 μM	3.13
Epirubicin + **5** (12.5 μM)	2.09 μM	2.68
Epirubicin + **5** (25 μM)	2.55 μM	3.27
Epirubicin + **6** (12.5 μM)	0.97 μM	1.24
Epirubicin + **6** (25 μM)	1.02 μM	1.31

[a]Protection Index = $IC_{50\ \text{epirubicin + protector}}/IC_{50\ \text{epirubicin alone}}$

In order to elucidate the mechanisms underlying the observed cytoprotection the DPPH radical-scavenging activities of compounds **1** together with the previously isolated benzophenones (**2**-**5**), and the xanthone genistein (**6**) were established. The results show that all compounds exhibited DPPH radical-scavenging activity compared to the control ($p < 0.05$) (Fig. (**3**)). Interestingly, the obtained results did not correlated well with the observed cytoprotective effects. Among the studied substances, the highest activity was observed for hypericophenonoside (**2**) (63,0 %) and annulatophenone (**3**) (79.8 %), while the remaining compounds were less active. The DPPH radical-scavenging activity of **3** was similar to that of the positive controls ascorbic acid (83.2 %) and BHA (84.2 %).

Furthermore, in an attempt to furthermore unravel mechanistically the established cytoprotection, the abilities of the tested compounds to modulate the levels of reduced glutathione (GSH) levels in K-562 cells were evaluated. Treatment with compounds **1**, **4** and **5** resulted in a significant elevation of cellular GSH levels (Fig. (**4**)), whereas the aglycones **3** and **6** lacked any significant effects in this respect (data not shown).

Finally, the effects of epirubicin, compounds **1**, **4** and **5** and combinations thereof against the colony-forming ability of murine bone marrow stem cells in semi-solid medium were evaluated. As evident from the results presented on (Fig. **3**) epirubicin (1.25 μM) significantly inhibited the clonogenicity of bone marrow cells following 24 h exposure. All of the benzophenones were absolutely devoid of anticlonogenic activity themselves (data not shown). The concomitant administration of compound **1** decreased the detrimental effects of the anthracycline epirubicin towards bone marrow colony forming units, following 24 h exposure, whereas neither **4** nor **5** exerted significant cytoprotective effects on this model system (Fig. (**5**)). In order to thoroughly characterize the cytoprotection afforded by **1**, the LTBMCC were analyzed by phase-contrast microscopy at the end of the exposure (Fig. (**6**)). Co-administration (epirubicin+**1**) ameliorated the cytotoxic effects of epirubicin as evidenced

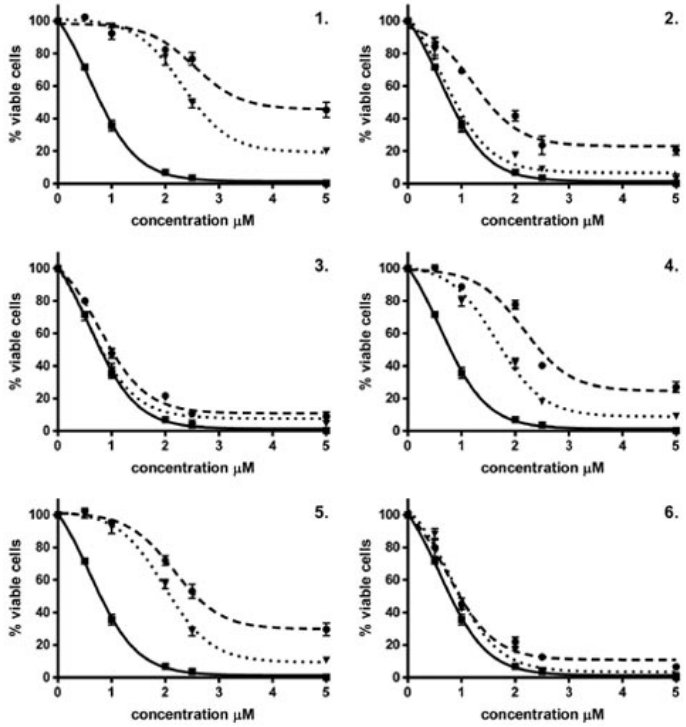

Fig. (2). Cytoprotective effects of the tested compounds (**1**, **2**, **3, 4**, **5** and **6,** as indicated on the graphs) against epirubicin-induced cytotoxicity in K-562 cells. The concentration-response curves represent the effexts of epirubicin alone (■), or in combination with the corresponding compound at 12.5 μM (●) or 25 μM (▼), after 72 h exposure (MTT-dye reduction assay).

by the less pronounced features of cell injury encountered in this treatment group as compared to the LTBMCC treated with the anthracycline alone.

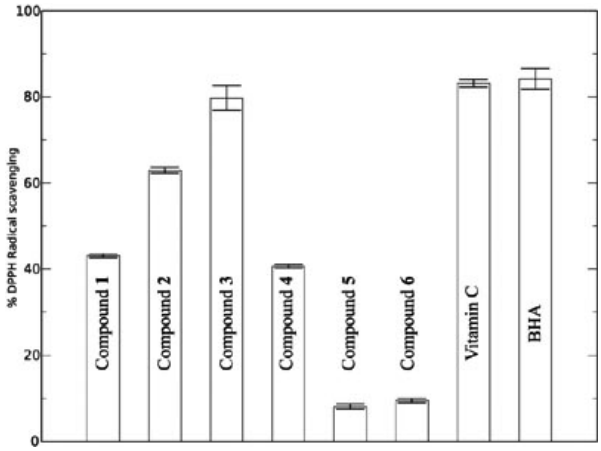

Fig. (3). Comparison of the DPPH radical-scavenging activity of investigated compounds to ascorbic acid and BHA.

Plant-derived polyphenols are well-known for their antioxidant effects [9], which gave us reason to examine the ability of the benzophenones isolated from *H. annulatum* to ameliorate the cytotoxic effects induced by antineoplastic agents *in vitro*. The chosen model compound epirubicin, though endowed by a complex mode of action, has the propensity to modify DNA structure and to induce a free radical generation and an oxidative stress [32,33]. The results from the cytoprotection assessment revealed that the glycosides substantially reduce the detrimental effects induced by epirubicin in K-562 cells, a finding which could be generally ascribed to the observed GSH level elevation following the treatment of these cells with compounds **1**, **4**

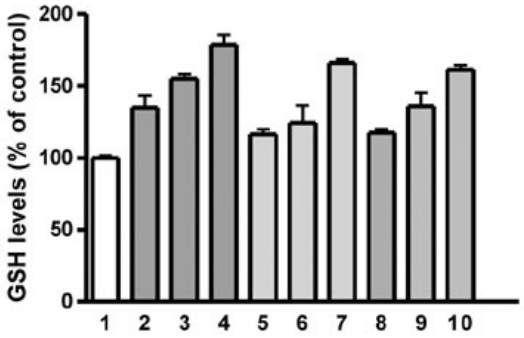

Fig. (4). Effects of **1**, **4** and **5** on the glutathione levels in K-562 cells after 48 h exposure. Neoannulatophenonoside **1** at 6.25 μM (column 2), 12.5 μM (column 3) and 25 μM (column 4); annulatophenonoside **4** at 6.25 μM (column 5), 12.5 μM (column 6) and 25 μM (column 7); and acetylannulatophenonoside **5** at 6.25 μM (column 8), 12.5 μM (column 9) and 25 μM (column 8); vs. untreated control (column 1).

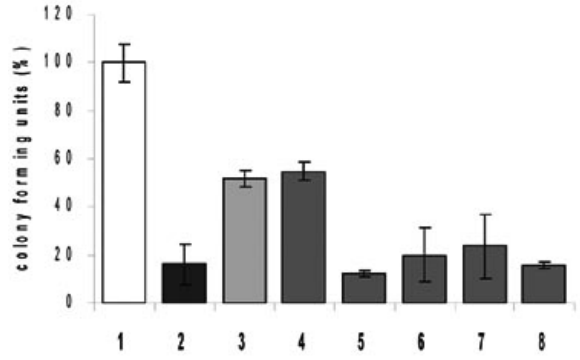

Fig. (5). Effects of **1**, **4** and **5** on the anticlonogenic activity of epirubicin in LTBMCC. Colony forming units (% of control) after treatment with 1.25 μM epirubicin alone (column 1), or in combination with: neoannulatophenonoside **1** (12.5 μM (column 3) and 25 μM (column 4)), annulatophenonoside **4** (12.5 μM (column 5) and 25 μM (column 6)) and acetylannulatophenonoside **5** (12.5 μM (column 7) and 25 μM (column 8)), vs. untreated control (column 1).

and **5**. GSH, which is the most abundant non-protein thiol in mammalian cells, plays a crucial role for the detoxification of free radicals and electrophilic noxae [32,34]. Conversely, it is firmly established that the agents mimicking the actions of glutathione and or modulating its cellular levels could greatly govern the cellular susceptibility to chemotherapeutic agents at all [34].

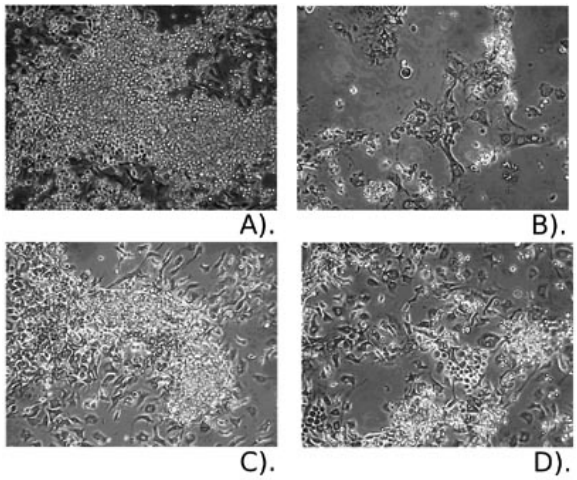

Fig. (6). Phase-contrast microscopic imaging of murine LTBMCC after treatment with 1.25 μM epirubicin (B), 25 μM compound **1** (C), 1.25 μM epirubicin + 25 μM compound **1** (D), versus untreated control (A).

The structure activity relationship analysis shows that the presence of a sugar moiety is a crucial structural prerequisite for the occurrence of cytoprotective activity, at least in the employed test system as both the aglycones **3** and **6** were the least active agents evaluated. Furthermore, the marginal cytoprotective effects of **2** could be explained by the fact that it is easily converted into the inactive **6** [22].

Interestingly, the compounds which showed considerable free radical scavenging potential failed to demonstrate significant cytoprotective activity against the epirubicin-induced cellular toxicity. This is most likely an outcome of the sophisticated mode of action of epirubicin, whereby the oxidative stress is only one of the underlying mechanisms and on the other hand it should be noted that the antioxidant assay only reveals one of the possible mode of antioxidant activity peculiar to polyphenols at all. Apart from their ability to act as scavengers, they are also readily oxidized due to their low redox potentials and furthermore the tested compounds are metal chelators as well [9]. It is firmly established that the toxicity of anthracyclines upon normal tissues is, to a great extend mediated by the production of reactive oxygen species during a Fe^{3+}- dependent process [32,33]. Thus a number of antioxidants have been shown to ameliorate the detrimental effects of anthracyclines on non-malignant cells, and amongst them the iron chelating agent endoxan has been utilized clinically for this objective [5,34].

The encountered protection against the epirubicin-induced inhibition of bone-marrow cell clonogenicity by co-administration of neoannulatophenonoside **1** could be ascribed to the antioxidant and/or metal chelating properties of the latter. Taken together our experimental data indicate that the benzophenone glycosides isolated from *H. annulatum* show a substantial cytoprotective potential against the toxic effects induced by epirubicin necessitates further and more detailed pharmacological evaluation as possible chemoprotective/ radioprotective agents.

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