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Eco-Friendly Synthesis of Lipophilic EGCG Derivatives and Antitumor and Antioxidant Evaluation

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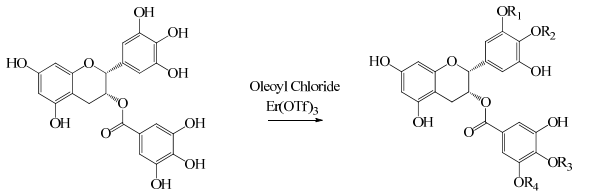
Epigallocatechin gallate (EGCG) was structurally modified to obtain EGCG fatty acid esters using an eco-friendly method employing $Er(OTf)_3$ in 2-MeTHF as the catalyst. The products were purified in short times on a large scale using VERSAFLASH HTFP station on octadecyl-functionalized silica gel. The same Lewis acid showed to be a good catalyst for the synthesis of EGCG peracetated. The EGCG derivatives were assayed for their antioxidant capacity by DPPH assay and by ROS formation using the SH-SY5Y cell line.

Keywords: EGCG, EGCG fatty acid esters, Log P, Antioxidants, Neuroblastoma cells.

Green tea is produced without fermentation from the leaves of *Camellia sinensis* belonging to Theaceae family. Green tea is rich in polyphenols, and its major biologically active components are catechins, being (-)-epigallocatechin-3-gallate (EGCG) the most abundant and most effective [1].

EGCG has shown many health benefits against bacterial and viral infections, inflammation, and cardiovascular disorders. Recently, the anti-obesity and anti-diabetic effects of green tea extracts, in terms of mechanism of its action, were studied and elucidated [2, 3]. It has also been reported that green tea consumption is associated to neuroprotective effects on Parkinson and Alzheimer's diseases. EGCG protects dopaminergic neurons against superoxide anions, free radicals and apoptosis induced by 6-hydroxydopamine (6OH-DA). Furthermore, EGCG prevents DNA damages through its metal chelating activity and interacts specifically with brain proteins such as "αS" to inhibit toxic fibrils aggregation [4]. EGCG binds amyloid-beta (Aβ) peptide and inhibits the toxic intracellular formation of oligomers [2]. Its antineoplastic effect against various types of cancers including liver, prostate, breast, and colon has also been reported [5, 6]. The cancer-preventive effect seems to be related to the presence of the phenolic hydroxyl groups in its structure [7, 8]. Tea polyphenols act like antioxidants *in vivo* but this activity is lower when compared to *in vitro* tests [9]. Moreover, catechins are chemically unstable in alkaline and oxidative media, and they undergo massive phase II metabolism transformations. EGCG can be modified structurally to improve its lipophilicity, expand its application in lipophilic media, and enhance its cellular absorption *in vivo*. The fully acetylated derivative (EGCG-P) may act as a pro-drug, leading to higher bioavailability than EGCG itself [10]. Moreover, EGCG peracetated and EGCG fatty acid monoester derivatives may improve the anticancer and neuroprotective activity in an alkyl chain length-dependent manner [11]. It has also been reported a further modification of EGCG under enzymatic catalysis, to afford butanoyl (EGCG-C4), octanoyl (EGCG-C8), and palmitoyl (EGCG-C16) monoesters derivatives. Their antitumor activities were investigated in *vitro* and *in vivo* and compared to free EGCG. Of the examined compounds, EGCG-C16 proved the most powerful inhibitor, suppressing colorectal cancer growth [12]. These derivatives showed excellent antiviral activity in inhibiting HCV protease and α-glucosidase, which were not significant for EGCG [13]. Furthermore, they are important antioxidants for use in food and cosmetic, and pharmaceutical industries [14].

In the context of our ongoing studies on Er(III) catalysis in mild, non-dry reaction conditions [15-17], and in continuation of our research program to develop environmentally friendly reactions [18] we report here the esterification of EGCG using $Er(OTf)_3$ as catalyst. $Er(OTf)_3$ is easy to handle, recyclable and is one of the cheapest commercially available lanthanide triflate derivatives [19]. Preliminary experiments were carried out on the model reaction between EGCG and oleoyl chloride using Er(III) as catalyst (Scheme 1) to obtain EGCG-monoester derivatives.



EGCG

Mixture of EGCG-monoester derivatives (a-d)

a: R_4=H; R_2=H; R_3=H; R_1=Oleoyl;
b: R_1=H; R_4=H; R_3=H; R_2=Oleoyl;
c: R_1=H; R_2=H; R_4=H; R_3=Oleoyl;
d: R_1=H; R_2=H; R_3=H; R_4=Oleoyl;

Scheme 1: Esterification of EGCG with oleoyl choride using $Er(OTf)_3$ as a catalyst.

The reaction was tested in water in ultrasonic output and in a Synthos 3000 microwave (Table 1, entries 1-3) obtaining poor results in terms of yield. Low product yields were obtained using acetonitrile as the solvent and performing the reactions with different molar ratios of EGCG/acyl chloride (1/2-1/5) (Table 1, entries 4-7).

Table 1: Optimization of the synthetic method [a]

Entry	Solvent	Temperature °C	EGCG/OleoylCl Molar ratio	Time min.	Yield[b] %
1	H_2O	r.t.	1/2	90	0
2[c]	H_2O	r.t.	1/2	30	0
3[d]	H_2O	60	1/2	20	0
4	AcCN	r.t.	1/2	180	0
5	AcCN	r.t.	1/5	180	0
6	AcCN	40	1/5	180	5
7[d]	AcCN	60	1/5	20	10
8	2-MeTHF	r.t.	1/2	180	5
9	2-MeTHF	60	1/2	120	20
10[d]	2-MeTHF	60	1/2	20	70
11[d]	2-MeTHF	60	1/1.2	20	70
12[e]	2-MeTHF	60	1/1.2	20	0
13[f]	2-MeTHF	60	1/1.2	20	20

[a] General reaction conditions: EGCG (0.6 mmol.), oleoyl chloride (2, 1.2 equiv.) and $Er(OTf)_3$ (10 mol %) were dissolved in 3 mL of solvent. [b] Percent yield calculated from HPLC data . [c] The reactions were conducted in ultrasonic output, 2 x 240 att/period, 35 kHz (FRITSCH). [d] The reactions were conducted in a Synthos 3000 microwave oven (Anton-Paar).[e] $Er(OTf)_3$ 5 mol%. [f] No catalyst used.

Good results were obtained using 2-MeTHF as the solvent under MW irradiation and using 1.2 mmol of oleoyl chloride (Table 1, entry 11). A mixture of four regioisomers in 70% yield was obtained and each EGCG-O-acyl derivative possesses acyl group in one of the hydroxyl groups at the B- or D-ring (Scheme 1). The optimized methodology was applied for the synthesis of different *O*-acyl derivatives of EGCG such as EGCG-C4, EGCG-C6, EGCG-C16 and EGCG-C18 (Table 2).

Table 2: Synthesis[a] and lipophilicity[b] of mono-*O*-acyl EGCG derivatives.

Entry	R	Product	Yield[c] %	log P
1	$CO(CH_2)_2CH_3$	EGCG-C4	41	2.83
2[c]	$CO(CH_2)_4CH_3$	EGCG-C6	53	3.51
3[d]	$CO(CH_2)_{14}CH_3$	EGCG-C16	65	8.13
4	(*cis*) $CO(CH_2)_7CH{=}CH(CH_2)_7CH_3$	EGCG-C18	70	8.65

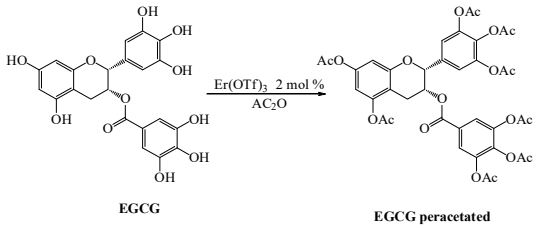
[a] General reaction conditions: EGCG (0.6 mmol), acyl chloride (1.2 equiv.) and $Er(OTf)_3$ (10 mol %) were dissolved in 3 mL of solvent. [b] The lipophilicity (logP) of each compound was calculated using a recently proposed method [20]. [c] Percent yield calculated from HPLC data.

We optimized the process in terms of purification in order to obtain the pure products in short time and with extreme efficiency. The purification using silica gel as a stationary phase leads to a partial degradation of the monoacyl derivative thus lowering the yield.

The use of a reversed-phase chromatography on C18 SPE cartridges enabled to obtain a no degraded product in short times, nevertheless the pure product was not entirely obtained. When the purification was performed on a Supelco VERSA FLASH HTFP station on octadecyl-functionalized silica gel [21], the residue was purified by a peristaltic flow in short times and the product was obtained in excellent yield.

Once assessed the successful use of Er(III) as a catalyst in the preparation of EGCG oleate derivatives, we tested its behaviour in the synthesis of EGCG peracetated. Several methods have been reported for the preparation of acetyl derivatives using various metal salts, such as $Mg(ClO_4)_2$, $Zn(ClO_4)_2$ x $6H_2O$ [22], triflate catalysts or stoichiometric reagents. Nonetheless, most of these reactions were performed in conventional organic solvents.

Our research interests cover a range of topics including the development of environmentally friendly catalytic methods for the strategic protection/deprotection of functional groups [23] and some of them concern the acylation of alcohols and phenols using Er(III) as a catalyst [24]. We herein report also a simple and eco-friendly method for the synthesis of EGCG peracetated using acetic anhydride as the acylating agent under solvent free conditions at room temperature (Scheme 2).



Scheme 2: Synthesis of EGCG peracetated with $Er(OTf)_3$.

The progress of the reaction was monitored by TLC and the formation of the corresponding peracetated derivative was observed after 2 h.

The antioxidant and biological activity of the obtained lipophilic derivatives were tested and compared with peracetated EGCG and free EGCG. A high Log P value is a good index of ability to pass across the cell membrane, thus for this reasons oleate monoester derivative was selected for biological testing (see Table 2). DPPH assay [25] was used as a rapid thin layer chromatography screening method to evaluate the antioxidant activity (Table 3).

Table 3: EGCG, EGCG-C18 and EGCG peracetated free radical-scavenging activity measured using the DPPH Assay.

entry	Compound	IC_{50}±SD [μM][a]
1	EGCG	6.5±0.55
2	EGCG-C18	12.9±0.25
3	EGCG-peracetated	29.6±0.27

[a]Data are expressed as the means ±SD of three independent observations. $^*p < 0.05$

Recently, Reuter *et al*. [26] clearly showed the role of reactive oxygen species (ROS) in various phases of tumorigenesis. We assessed the ROS scavenging capacities of the synthesized molecules, by determining their ROS levels in a biological environment using the SHSY-5Y tumor cell line (Figure 1). SH-SY5Y cells were exposed to extracellular ROS attack by 6-OHDP (6-Hydroxydopamine) and pretreated with different concentrations of EGCG, EGCG oleate and EGCG peracetated (1, 0.5, 0.1 and 0.05 μM). Cell proliferation tests were performed on the SH-SY5Y cancer cells of neuroblastoma using a SRB assay protocol (Figure 2).

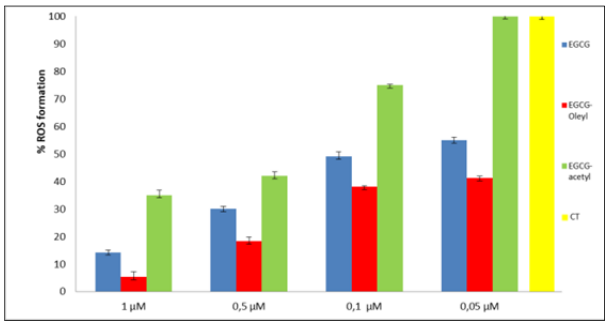


Figure 1: ROS formation: SHSY-5Y cells were exposed to the extracellular ROS attack by 6-OHDP 25 μM (and pretreated with EGCG; EGCG oleate and EGCG peracetated to concentrations 1, 0.5, 0.1 and 0.05 μM. Data expressed as means ±SD of three independent observations. $^*p < 0.05$.

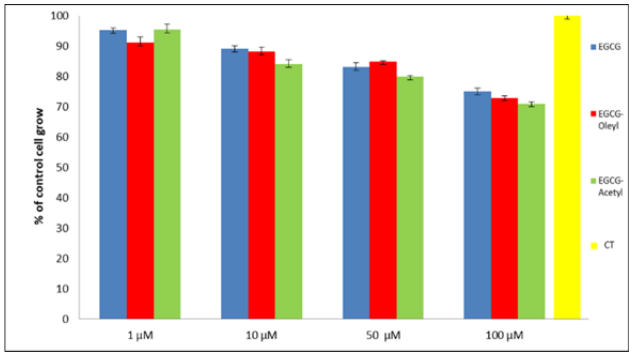


Figure 2: SRB Assay: SRB Assay for determination % of control cell grow. Data expressed as means ±SD of three independent observations. $^{*}p < 0.05$.

Exposure of SHSY-5Y cells to 6-OHDP (25 µM) resulted in a significant increase in intracellular ROS production compared with untreated cells. In the presence of EGCG oleate, ROS generation was significantly reduced, and a 41% reduction in ROS formation was observed at only 0.05 µM. The inhibition of ROS formation decreases with the reduction of the lipophilicity of EGCG derivatives, demonstrating the connection between lipophilicity, cellular bioavailability and antioxidant activity.

Experimental

General: ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker WM 300 NMR spectrometer and WM 75 NMR on samples dissolved in $CDCl_3$. Chemical shifts are given in parts per million (ppm) from tetramethylsilane as the internal standard (0.0 ppm). Coupling constants (*J*) are given in Hz. TLC were performed using silica plates 60-F264 on alumina, commercially available from Merck. Epigallocatechin gallate (EGCG), oleoyl chloride and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis of EGCG-Acyl: To a 2-MeTHF (50 mL) suspension of EGCG (500 mg, 1.09 mmol) acyl chloride (1.31 mmol) and $Er(OTf)_3$ (10% mol) were added. The mixture was reacted under stirring for 20 min in a Synthos 3000 microwave oven (Anton-Paar). TLC monitored the disappearance of EGCG and, after completion, the crude product was purified by Supelco VERSA FLASH HTFP station to give the mixture of EGCG monoester derivative regioisomers. The analyte was eluted with a linear water/methanol gradient, starting from 0% methanol, up to 10% methanol. The collected fractions were monitored by HPLC.

EGCG-monobutyrate regioisomers (EGCG-C4)
^{1}H-NMR (500 MHz, $CDCl_3$): 0.96 (3H, t, J = 6.62 Hz, –CH_3 in 1a–1d); 1.60 (2H, m, –CH_2, 1a–1d), 2.22 (2H, m, –CH_2, 1a–1d), 2.83 (1H, m, H-4, 1a–1d), 2.96 (1H, m, H-4, 1a–1d), 5.00 (1H, m, H-2, 1a–1d), 5.46 (1H, m, H-3, 1a–1d), 5.94 (2H, m, H-6 e H-8, 1a–1d), 6.05 (2H, dd, CH=CH, J= 2.18 Hz), 6.47 (0.43H, s, 2' e 6', 1d), 6.50 (0.17 H, s, 2' e 6', 1c), 6.56 (0.17 H, s, 2' e 6', 1b), 6.74 (0.17 H, d, J = 2.04 Hz, 2' o 6' di 1b), 6.79–6.82 (7H, br s, –OH), 6.83 (0.23H, d, J = 1.96 Hz, 2' o 6', 1a), 6.85 (0.23H, s, 2'' e 6'', 1a), 6.94 (0.17H, s, 2'' e 6'', 1b), 6.95 (0.17H, s, 2'' e 6'', 1c), 7.10 (0.43 H, d, J = 2.53 Hz, 2'' o 6'', 1d), 7.49 (0.2 H, d, J = 8.5 Hz, 2'' o 6'', 1d). 1a:1b:1c:1d=23:17:17:43
^{1}H NMR (400 MHz, $CDCl_3$): 1.90 (3H, s, Me), 2.79 (3H, s, COMe), 7.20 (1H, d, J = 8.1 Hz, H-7)
^{13}C NMR (100 MHz DMSO-d_6): 8.9 (CH_3), 30.3 (CH_2), 51.9 (CH), 169.6 (C).

EGCG-monohexanoate regioisomers (EGCG-C6)
^{1}H-NMR (500 MHz, $CDCl_3$): 0.98 (3H, t, J = 6.62 Hz, –CH_3, 2a–2d); 1.63 (4H, m, –CH_2, 1a–1d), 2.24 (4H, m, –CH_2, 2a–2d), 2.89 (1H, m, H-4, 2a–2d), 2.96 (1H, m, H-4, 2a–2d), 5.02 (1H, m, H-2 di 2a–2d), 5.43 (1H, m, H-3 di 2a–2d), 5.90 (2H, m, H-6 e H-8 di 2a–2d), 6.03(2H, dd, CH=CH, J = 2.18), 6.46 (0.45H, s, 2' e 6' di 2d), 6.50 (0.45H, s, 2' e 6' di 2c), 6.52 (0.15H, s, 2' e 6' di 2b), 6.71 (0.15H, d, J = 2.04 Hz, 2' o 6' di 2b), 6.77–6.83 (7H, br s, –OH), 6.84 (0.25H, d, J = 1.96 Hz, 2' or 6' di 2a), 6.86 (0.25H, s, 2'' e 6'' di 2a), 6.93(0.15H, s, 2'' e 6'' di 2b), 6.98 (0.14 5H, s, 2'' e 6'' di 2c), 7.12 (0.45 H, d, J = 2.53 Hz, 2'' o 6'' di 2d), 7.51 (0.2 H, d, J = 8.5 Hz, 2'' o 6'' di 2d).
2a:2b:2c:2d=25:15:15:45

EGCG-monopalmitate regioisomers (EGCG-C16)
^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.8 Hz, –$COCH_2CH_2(CH_2)_{12}CH_3$, 4a–4d), 1.27 (26H, m, –$COCH_2CH_2(CH_2)_{12}CH_3$, 4a–4d), 1.67 (2H, tt, J = 14.6, 7.9 Hz,–$COCH_2CH_2(CH_2)_{12}CH_3$, 4a–4d), 2.58 (2H, m,–$COCH_2CH_2(CH_2)_{12}CH_3$, 4a–4d), 2.82 (1H, m, H-4 , 4a–4d), 2.97 (1H, m, H-4, 4a–4d), 5.03 (1H, m, H-2, 4a–4d), 5.49 (1H, m, H-3, 4a–4d), 5.98 (2H, m, H-6 and H-8, 4a–4d), 6.49 (0.4H, s, 2'e 6', 4d), 6.50 (0.14H, s, 2'and 6' , 4c), 6.56 (0.7H, s, 20 e 60, 4b), 6.74 (0.38H, d, J = 2.04 Hz, 2'or 6', 4a), 6.82 (0.38H, d, J = 1.96 Hz, 2' or 6', 4a), 6.82–6.70 (7H, br s, –OH), 6.88 (0.76H, s, 2''and 6', 4a), 6.90 (0.7H, s, 2'and 6', 4b), 6.92 (0.14H, s, 2''and 6'', 4c), 7.09 (0.2H, d, J = 1.96 Hz, 2'' or 6'', 4d), 7.21 (0.2H, d, J = 2.00 Hz, 2'' or 6'', 4d).
4a:4b:4c:4c=20:18:18:44

EGCG-monooleate regioisomers (EGCG-C18)
^{1}H-NMR (500 MHz, $CDCl_3$) 0.84 (3H, t, J = 6.62 Hz, –CH_3, 3a–3d); 1.08 (26H, m, –$(CH_2)_nCH_3$, 3a–3d), 1.22-2.14 (30 H, m, $(CH_2)_n$, 3a–3d), 2.82 (1H, m, H-4 , 3a–3d), 2.97 (1H, m, H-4, 3a–3d), 5.03 (1H, m, H-2, 3a–3d), 5.49 (1H, m, H-3, 3a–3d), 5.98 (2H, m, H-6 and H-8, 3a–3d), 6.08 (2H, dd, CH=CH, J = 2.18 Hz), 6.49 (0.4H, s, 2'and 6', 3d), 6.50 (0.14H, s, 2'and 6', 3c), 6.56 (0.7H, s, 2'and 6', 3b), 6.74(0.38H, d, J = 2.04 Hz, 2'or 6', 3a), 6.79–6.82 (7H, br s, –OH), 6.83 (0.38H, d, J = 1.96 Hz, 2'or 6', 3a), 6.85 (0.76H, s, 2'' and 6'', 3a), 6.94 (0.7H, s, 2''and 6'', 3b), 6.95 (0.14H, s, 2''and 6'', 6c), 7.10 (0.2H, d, J = 2.53 Hz, 2'' or 6'', 6d), 7.49 (0.2H, d, J = 8.5 Hz, 2'' or 6'', 3d).
3a:3b:3c:3d=40:10:10:40

Synthesis of EGCG-peracetated: EGCG (500 mg, 1.09 mmol) was reacted for 2 h at room temperature with an excess of acetic anhydride (15 mL) in the presence of 2 mol % of $Er(OTf)_3$ (0.02 mmol). At the end of the conversion, 15 mL of MeOH was added to the mixture and stirred for further 1 h. Solvent was removed under vacuum, and the residue was solubilized in CH_2Cl_2. The mixture was extracted with a saturated solution of $NaHCO_3$ until complete elimination of the acetic acid; the collected organic phases were dried on Na_2SO_4, and filtered, and the solvent was evaporated under pressure. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 8:2, as eluent) (75% yield, white crystal).
^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.21 (CH_3-Ac × 2), 2.23 (CH_3-Ac), 2.25 (CH_3-Ac), 2.26 (CH_3-Ac × 3), 2.27 (CH_3-Ac), 2.98 (dd, 1H, J = 18.0, 2.3 Hz, H-4b), 3.04 (dd, 1H, J = 18.0, 4.6 Hz, H-4a), 5.16 (br s, 1H, H-2), 5.62 (m, 1H, H-3), 6.59 (d, 1H, J = 2.2 Hz, H-8), 6.71 (d, 1H, J = 2.2 Hz, H-6,), 7.24 (s, 2H, H-2′, 6′), 7.60 (s, 2H, H-2″, 6″).
^{13}C- NMR (125 MHz, $CDCl_3$): δ 20.1-21.1 (CH_3-Ac × 8), 25.9 (C-4), 68.0 (C- 3), 76.5 (C-2), 108.1 (C-8), 109.0 (C-6), 109.4 (C-4a), 118.8 (C- 2″, 6″), 122.4 (C-2′, 6′), 127.5 (C-1″), 134.4 (C-4′), 135.1 (C-1′), 139.0 (C-4″), 143.3 (C-3″, 5″), 143.5 (C-3′, 5′), 149.7 (C-7),

149.8 (C-5), 154.8 (C-8a), 163.5 (CO), 166.2 (CO-Ac), 166.7 (CO-Ac), 167.4 (CO-Ac × 2), 167.5 (CO-Ac × 2), 168.4 (CO-Ac), 168.8 (CO-Ac).

DPPH radical scavenging activity: DPPH radical-scavenging activity of the samples was assessed by Gulcin method [26]. The 1,1-diphenyl-2-picrylhydrazyl radical solution was prepared by dissolving an appropriate amount of DPPH in MeOH to give a concentration of 100 μM. The DPPH radical solution (1 mM; 1mL) was added to a solution (1 ml) of the compound to be tested in MeOH at various concentrations (100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.124 μM, 1.562 μM, 0.78 μM). The mixture was shaken and incubated at room temperature in the dark for 40 min. The decrease in the absorbance of the resulting solutions was then measured spectrophotometrically at ν 517 nm. All measurements were made in triplicate [27]. The negative control (blank) is MeOH (2 mL) and the DPPH radical solution (2 mL), the positive control comprising the reference anti-oxidant in MeOH and DPPH radical solution. Inhibition of free radical DPPH in percentage was calculated as follows:

$$Inhibition\ (\%) = [(Control - Test) / Control] \times 100$$

Where Control is the absorbance of negative control (DPPH solution) and Test is the absorbance of compounds at different concentrations.

Sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting the inhibition percentage against sample concentration.

***Cell culture*:** The human SH-SY5Y neuroblastoma cell line was purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Ltd.) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM L-Glutamine, and 5 mg/ml penicillin/streptomycin. Cells were kept in incubation at 37°C in an atmosphere of 5% CO_2. After two passages, SH-SY5Y cells were plated at the density of $2x10^4$ cells/well in a 96-well microplate for the SRB and reactive oxygen species (ROS) assays.

***Sulforhodamine B cell proliferation assay*:** The *in vitro* cytotoxicity of the EGCG and EGCG derivatives against SH-SY5Y cells were compared using a SRB assay [28]. In the present screening experiment, the cells were inoculated in 96-well microtiter plates in 200 μL at $2x10^4$ cells/well and allowed to attach overnight. Meantime, a stock solution of the drugs in dimethylsulfoxide 0.1 M was prepared. An aliquot of 1.2 μL of this stock solution was then diluted to 0,1 μM, 1 μM 10 μM and 100 μM with the colture medium. After removing the cell media, the drugs prepared as described above were added to achieve a final volume of 200 μL for each well. After the addition of the different drug concentrations (200 μL), the plates were incubated under standard conditions for 24 h; the assay was terminated by the addition of cold TCA. After discarding the supernatant, the cells were fixed in situ gently via the addition of 100 μL of cold TCA 10% (w/v) and incubated for 60 min at 4 °C. Next, the plates were washed five times with double distilled water (DDW) and then air-dried. After adding Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid to each well, the plates were incubated at room temperature for 30 min. Upon appearance of the staining, the unbound dye was collected and the plates were washed five times with 1% acetic acid to remove residual dye and the air-dried. The bound stain was subsequently eluted with 200 μL of 10 mM unbuffered Tris-based solution (pH 10.5, Sigma) and the plate was shaken for 15 min. The absorbance was measured at 492 nm using a Spectra-Max-190 (Molecular Devices, Sunnydale, USA) microplate reader [29]. The plate-by-plate examination of the test wells relative to control wells was employed to determine percent growth, and the ratio of the average absorbance in the test well to the average absorbance in the control wells × 100 was determined.

$$Growth\ (\%) = (Test / Control) \times 100$$

ROS Formation. The DMSO drug solution was added to cells (plated at $2x10^4$ cells/well dilution in 250 μL, in a black, flat-bottom 96-well plate (Thermo Fisher Scientific) and incubated overnight) at the appropriate concentrations diluted in 100 μL fresh culture medium. After 2.5 h, 50 μL of 2,7-dichlorofluorescin diacetate (DCFH-DA, Sigma, 5000 μM stock solution in EtOH) was added to the wells to achieve a final concentration of 5 μM and the plates were wrapped in aluminum foil and incubated at 37°C. The loading took 30 min. Afterwards, 100 μL of 6-OHDP (6-hydroxy dopamine, Sigma, 10000 μM stock solution in fresh medium) was added to achieve a final concentration of 25 μM [30]. At this concentration, the 6-OHDP incubation resulted no reduction in cell viability. After wrapping the plates in aluminum foil again, the plates were incubated for an additional 24 h period. DCF fluorescence was read at 530 nm. ROS formation data were calculated with the following equation :

$$ROS\ formation\ (\%) = [(M.F.U.\ drug\ DMSO + 6\text{-}OHDP\ cells) - (M.F.U.\ drug\ DMSO\ blank) - (M.F.U.\ DMSO\ cells)/ (M.F.U.\ 6\text{-}OHDP\ cells) - (M.F.U.\ DMSO\ cells)] \times 100$$

Statistical analysis: The results are expressed by mean ± S.E.M. from at least three independent experiments. For statistical comparisons, quantitative data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-test according to the statistical program SigmaStat1 (Jandel Scientific, Chicago, IL, USA). A *p*-value less than 0.05 was regarded as significant.

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