Flavan-3-ols from the Leaves of Malaysian *Uncaria longiflora* var. *pteropoda* (Miq.) Ridsd.

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Abstract

A novel flavonoid, (-)-2*R*,3*R*-3,5,4'-trihydoxyflavan-[6,7:5",6"]-2"-pyranone, named uncariechin (**1**), was isolated from the methanolic extract of the leaves of *Uncaria longiflora* var. *pteropoda* (Miq.) Ridsd. along with the known (-)-*epi*afzelechin (**2**) and (-)-*epi*catechin (**3**), methyl 4-hydroxybenzoate and 4-hydroxybenzaldehyde, four pentacyclic oxindole alkaloids, isopteropodine, pteropodine, uncarine F and isopteropodic acid, previously found in the stems, and two coumarins, scopoletin and 3,4-dihydroxy-7-methoxycoumarin. Structures of the compounds were elucidated by 1D and 2D NMR, FTIR, UV, MS, and experimental as well as calculated electronic circular dichroism (ECD) data. Compounds **2** and **3** were evaluated for their neurotoxic and neuroprotective properties against differentiated SH-SY5Y neuroblastoma cell lines using the MTS assay. Compounds **2** and **3** did not show any neurotoxic effects but showed strong protective potential against hydrogen peroxide-induced neurotoxicity with maximum cell viability at a concentration of 1 μM.

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1. Introduction

We have previously reported the biological activity of the genus *Uncaria*, a genus belonging to the Rubiaceae family (Ahmad et al., 2011). The genus comprises thirty-four species, which are mainly shrubby woody climbers distributed in tropical regions, including Southeast Asia, Africa and Southeast America (Risdale, 1978). *U. longiflora* var. *pteropoda* is one of the fourteen species found in Malaysia (Risdale, 1978). Our previous work on the woody stem extracts of this plant led to the isolation of two new heteroyohimbine-type oxindole alkaloids, namely, rauniticine-*allo*-oxindole B and rauniticinic-*allo* acid B along with five of their stereoisomers (Salim et al., 2011). We have also reported on the chemotaxonomic significance of the pentacyclic oxindole alkaloids in the species (Salim and Ahmad, 2011). In this paper, we report the isolation and characterization of a novel flavonoid (-)-2*R*,3*R*- 3,5,4'-trihydoxyflavan-[6,7:5",6"]-2"-pyranone, named uncariechin (**1**), along with (-)-*epi*afzelechin (**2**) and (-)-*epi*catechin (**3**) from the methanol extract of the leaves as well as methyl 4-hydroxybenzoate and 4-hydroxybenzaldehyde, four pentacyclic oxindole alkaloids, isopteropodine, pteropodine, uncarine F and isopteropodic acid previously found in the stems (Salim and Ahmad, 2011) and two coumarins, scopoletin and 3,4-dihydroxy-7-methoxycoumarin (Abu-Eittah and El-Tawil, 1985) by spectroscopic techniques including FTIR, UV and 1D and 2D NMR spectroscopy, MS, electronic circular dichroism (ECD) measurements and calculations. This is the first time the flavonoid composition of this plant has been described. In view of the potential of catechins as therapeutic cytoprotective agents for the treatment of neurodegenerative and other diseases (Mandel and Youdim, 2004), we investigated the neurotoxic and neuroprotection properties of the flavan-3-ols isolated against differentiated SH-SY5Y neuroblastoma cell line.

2. Results and Discussion

Compound **1** was obtained from the MeOH extract of the leaves of *U. longiflora* var. *pteropoda* as pale yellow crystals (m.p. 249–250 ºC). It was observed on TLC as a bluish fluorescent spot under UV light (365 nm) and on UV measurement it showed absorption

maxima at 276, 248, 212 and 195 nm suggesting the presence of a flavan moiety (Merken et al., 2000). HRESI-MS indicated a molecular formula of $C_{18}H_{14}O_6$ and twelve degrees of unsaturation. The IR spectrum showed characteristic absorption bands for free hydroxyl groups (3435 cm^{-1}), conjugated lactone carbonyl stretching (1685 cm^{-1}), a cyclic ether group (1522 cm^{-1}) and methylene group bending (1457 cm^{-1}) .

The aromatic region of the ^{1}H NMR spectrum of compound **1** showed a pair of *ortho*-coupled proton resonances at δ 7.44 (2H, d, *J* = 8.4 Hz, H-2', H-6') and at δ 6.88 (2H, d, *J* = 8.7 Hz, H-3', H-5') for a *para*-substituted ring B of a flavonoid-type compound. A hydroxyl group was placed at C-4'. These assignments were supported by the COSY spectrum which showed correlations between the H-2' and H-3' and between H-5' and H-6' resonances as well as between the H-6' and H-2 resonances of ring C. For ring C, the COSY spectrum showed coupling between resonances at δ 5.17 (H-2), δ 4.38 (H-3), and δ 2.99–2.94 (2H-4). The chemical shift of H-3 indicated the presence of a hydroxyl group at this position establishing the presence of a flavan-3-ol. The 2H-4 proton resonances showed correlations in the HMBC spectrum with the C-5 (δ 114.78), C-9 (δ 160.15) and C-10 (δ 103.89) resonances. A singlet at δ 6.42 indicated a single proton on ring A. HMBC correlations between this resonance and the C-9 and C-10 resonances indicated this proton was at C-8 (δ 94.45). The H-8 resonance also showed HMBC correlations with the oxygenated C-7 resonance at (154.81) and a resonance at δ 102.08 which was ascribed to C-6.

Three carbon resonances remained to be assigned, a lactone carbonyl resonance (δ 160.55) and two alkene resonance (δ 138.64 and δ 109.71). The corresponding ^{1}H NMR resonances occurred at δ 8.06 (d, *J* = 9.6 Hz, H-4") and δ 6.07 (d, *J* = 9.6 Hz, H-3"). The HMBC spectrum was used to determine whether a linear or angular pyranocoumarin was present. ^3J correlations were seen between the H-4" resonance and C-5, C-7 and C-2" resonances. The molecular formula indicated the need for a fused ring and a pyranone ring was indicated. In the NOESY experiment run in DMSO, a correlation was observed between the H-4" and the 5'-OH (δ 6.03) proton resonances also confirming that the attachment was linear.

The relative configuration of compound **1** was established using a NOESY experiment and a model. The H-2 proton resonance showed correlations in the NOESY spectrum with H-

3 and one of H-4 proton resonances, allowing for the placement of ring B and the 3-OH on the same face of the molecule. A $J_{2,3}$ coupling constant of <1 Hz further confirmed a *cis* relationship for H-2 and H-3 in compound **1** (Friedrich and Galensa, 2002). The flavan-3-ols have two stereocenters and therefore four possible diastereomers, (2*R*,3*S*)-*trans*, (2*S*,3*R*)-*trans*, (2*R*,3*R*)-*cis* and (2*S*,3*S*)-*cis* are possible. A *cis* relationship between ring B and the 3-OH group would support a (*2R,3R*) or a (*2S,3S*) configuration (Friedrich and Galensa, 2002). Firstly, the absolute configuration of the known flavan-3-ols (-)-*epi*afzalechin (**2**) and (-)-*epi*catechin (**3**) were confirmed. As for **1**, a $J_{2,3}$ coupling constant of <1 Hz suggested a *cis* configuration for both **2** and **3** for a (*2R,3R*) or a (*2S,3S*) conformation. The measured optical rotation for compounds **2** and **3** were -151 and -125, respectively, supporting a (2*R*,3*R*) configuration (Nanjo et al., 1996). According to Slade *et al*., (2005), flavan-3-ols are characterized by two phenyl chromophores whose UV absorption bands are between 200 and 240 and between 260 and 280 nm giving fingerprint ECD Cotton effects at the respective wavelengths. A 2*R*,3*R*-*cis* configuration will show two negative Cotton effects at these wavelengths. To confirm this, experimental ECD analyses for **2** and **3** were carried out in which two negative Cotton effects at *ca*. 220 nm and *ca*. 270 nm were observed, consistent with a 2*R*,3*R*-*cis* configuration of the compounds. Thus, the absolute configuration of these compounds were established as (-)-2*R*,3*R*-*epi*catechin and (-)-2*R*,3*R*-*epi*afzalechin.

To determine the absolute configuration of **1** and investigate the effect of the pyranone moiety on its CD spectrum, both experimental and calculated ECD analyses were carried out. The latter was done *via* a systematic conformational search of the (2*R*,3*R*) isomer with the Spartan08 program using molecular mechanics force field (MMFF) calculations. This generated 12 conformers from which 8 conformers were under an energy cut off of 3 kcal/mol. An ECD analysis was then calculated for each of these conformers using time dependent density functional theory (TDDFT) at the B3LYP/6-31G (d, f) level built to Gaussian09 software (Ding et al., 2010). The calculated ECD spectra were Boltzmann weighted (BW) and compared to the experimental ECD spectrum of **1**. As shown in Fig. 1, the BW-ECD spectrum for the 2*R*,3*R* isomer of **1** showed a negative Cotton effect at *ca.* 220 and a small but distinct negative CE at ca. 270 nm. In addition, the calculated ECD spectrum also showed a positive CE at *ca.* 325 nm likely due to a $\pi \rightarrow \pi^*$ transition in the extended π-

system of pyranone moiety (Dastan *et al*., 2012). The two negative CEs observed indicated that the presence of the pyranone moiety fused to ring A of **1** did not affect the helicity of the molecule which was found to conform to Snatzke's helicity rule (Snatzke and Ho, 1971) as reported by Slade and co-authors (2005). A good match was found for the weighted ECD spectrum and the experimental ECD spectrum of compound **1** (Fig. 1) confirming a (2*R*,3*R*) absolute configuration leading to the establishment of compound **1** as (-)2*R*,3*R*-uncariechin. The structure of compound **1** is given in Figure 2.

In view of the reported cytoprotective activities of catechins (Mandel and Youdim, 2004) the neurotoxic and neuroprotective potential of the flavan-3-ols isolated in this study was evaluated. However, due to a limited amount of compound **1**, only compounds **2** and **3** were tested. The effect of pre-incubation with compounds **2** and **3** on the production of reactive oxygen species (ROS) by differentiated human neuroblastoma SH-SY5Y cell-line in the presence or absence of oxidative stress (H_2O_2) was evaluated at a concentration range of 1 nM–1 mM. Our data showed that both compounds **2** and **3** produced no neurotoxic effect on neuron phenotypic cells (differentiated SH-SY5Y; Figure 3) with a surprising increase in cell viabilty values exceeding 100% (negative control) at all concentrations suggesting the compounds' ability to proliferate the cells. We therefore investigated whether compounds **2** and **3** can exert a neuroprotective effect on the neuron phenotypic cells, SH-SY5Y. Hydrogen peroxide-induced neurotoxicity was used as a positive control (Godkar et al., 2006). Incubation with compounds for 2 h followed by exposure to 230 μM H_2O_2 showed an increase in cell viability to 75-88% and 77-85%, respectively, compared to a 52% cell viability for the positive control (Figure 4). Compounds **2** and **3** exhibited the highest cell viability of 88% and 85%, respectively, at a concentration of 1 μM with compound **2** displaying stronger neuroprotective potential than compound **3.** These findings are particularly relevant, since H_2O_2 is quantitatively the most important of the peroxides generated in brain cells (Dringen et al., 2005) and its intracellular accumulation can induce oxidative stress leading to neuronal apoptosis (Chandra et al., 2000).

In earlier studies, it has been reported that pre-treatment with *epi*catechin and 3-*O*-methyl-*epi*catechin attenuated neurotoxicity induced by oxidized low-density lipoprotein (oxLDL) in mouse-derived striatal neurons (Shroeter et al, 2001). Pre-treatment with the

compounds at a concentration of 30 µM prior to oxLDL administration led to cell viability of 90-93% as compared with 43% cell viability following treatment with oxLDL alone. In a related study, *epi*gallocatechin-3-gallate (EGCG) conferred protection against 6-OHDA-induced human neuroblastoma SH-SY5Y cell damage (Levites et al., 2002) where pretreatment for 15 min with the compound (0.1–10 µM) conferred significant protection against 6-OHDA neurotoxicity (38% cell viability at 50 µM). The authors also reported that EGCG showed maximal cell survival at 1µM (93%) with no effect up to 10 µM and a gradual decrease in cell viability at higher concentrations. Their results are in good agreement with our data for compound **3** (*epi*catechin) as shown in Figure 4 where the highest cell viability was also observed at 1 µM with a gradual decrease in cell viability at higher concentrations up to 100 µM. A similar trend was observed for compound **2** (*epi*afzelechin) for which, to the best of our knowledge, neurotoxic and neuroprotective potential have not been reported.

3. Experimental

3.1 General

TLC and PTLC were performed using pre-coated aluminium-backed supported silica gel 60 F_{254} (0.2 mm thickness) and glass supported silica gel 60 F_{254} (1.0 mm thickness), respectively. Flavonoids were detected on TLC stained with aluminium chloride ($AlCl_3$) reagent in which a positive result was indicated by the observation of yellow spots visualized under UV light at 365 nm. Column chromatography was carried out using silica gel 60, 70-230 mesh ASTM (Merk 7734) whereas radial chromatography was carried out using glass plates with Merck's silica gel Kieselgel 60 PF_{254} Merk Art 7749. Mass spectra were measured on an Agilent Technologies 6520 QTOF LC/MS equipped with a dual-ESI source and an Agilent Technologies LC system 1200 series, where the experiment for compounds **1**-**3** were run on negative mode, while the other compounds were run on positive mode. The ultraviolet (UV) spectra were obtained in methanol on a Shimadzu UV-Vis 160i. The infrared (IR) data was recorded on a Perkin Elmer model FT-IR spectrometer as KBr disks. Optical rotations were measured on a JASCO P1020 digital polarimeter. Melting points were determined using X-4 melting-point apparatus with microscope JM628 digital thermometer. The ECD spectrum for compounds **1, 2** and **3** were obtained on an Applied Photophysics Chirascan CD

spectrometer using a 5 mm cell and acetonitrile as the solvent. ^{1}H- and ^{13}C-NMR data for compound **1** were obtained in acetone-d_6 on Bruker 300 Ultrashield NMR spectrometer measured at 300 and 75 MHz, respectively.

3.2 Plant materials

Uncaria longiflora var. *pteropoda* stems and leaves were collected from Hutan Simpan Bangi, Selangor, Malaysia. The leaves and the stems of the plant were separated and the voucher specimens (HTBP 1336) were deposited at the Herbarium of Taman Botani Putrajaya, Malaysia.

3.3 Extraction and isolation of compounds

The leaves of *U. longiflora* var. *pteropoda* were cut into small pieces, air-dried and ground into a fine powder. The finely ground plant material was weighed, and extracted exhaustively with methanol at room temperature for 72 hours. The solvent was removed under reduced pressure to yield 550 g of crude extract which was successively triturated to afford 67 g, 72 g, 28 g and 362 g of hexane, chloroform, ethyl acetate and methanol extracts, respectively. The methanol extract (362 g) was then subjected to liquid-liquid partitioning between MeOH and Et_2O to remove the excess tannins. The dissolved portion was filtered through cotton wool and the solvent was evaporated to dryness using a rotary evaporator leaving 120 g of dark solid. Fractionation of the solid with vacuum liquid chromatography (VLC) using solvents of increasing polarity (DCM, EtOAc and MeOH) yielded five fractions (100 ml volumes).

Based on the TLC profiles, fractions 3 and 4 were found to contain a high density of flavonoids and were subsequently combined and subjected to further fractionation *via* column chromatography using DCM and MeOH with gradient elution to afford 11 fractions (F1-11) which was collected based on bands observed on the column. F7 to F9 were pooled and subjected to preparative thin layer chromatography (PTLC) using a solvent system of DCM:EtOAc (3:2) resulting in the isolation of pure compound **1** (17 mg). F10 afforded compound **2** (44 mg) upon purification with PTLC using a solvent system $CHCl_3$:MeOH (5:1). Employment of a different solvent system [$CHCl_3$:MeOH (33:7)] on the same fraction

successfully yielded a small amount of 3,4-dihydroxy-7-methoxycoumarin (4 mg). Similarly, compound **3** (100 mg) was purified from F11 by PTLC using $CHCl_3$: MeOH (62:13) as a solvent system. Fraction 2 was found to contain two distinct spots on TLC with UV visualisation at short wave length (254 nm) and upon PTLC development with solvent system Hexane:EtOAc (4:1) afforded methyl 4-hydroxybenzoate (8 mg) and 4-hydroxybenzaldehyde (4 mg).

The other five compounds were isolated from the chloroform extract (72 g) which was subjected to acid-base extraction to afford a crude alkaloid mixture (53 g). This mixture was chromatographed using VLC with increasing solvent polarity using hexane, DCM, EtOAc and MeOH to give nine fractions (100 ml volumes) of which those with the same TLC profiles were combined. Isopteropodine (3552 mg), pteropodine (2481 mg) and scopoletin (21 mg) were purified with column chromatography using solvent system Hexane:EtOAc (7:3) from pooled fractions F2 and F3. Preparative TLC of F5 using solvent system DCM:EtOAc (7:3) yielded uncarine F (43 mg) while centrifugal PTLC on F6 using solvent system $CHCl_3$:MeOH (20:1) led to the isolation of isopteropodic acid (55 mg). All known compounds were characterized by NMR spectroscopy and comparison with literature (Seki et al., 1993, Liu and Feng, 1993, Pouchert and Behnke, 1993, Prasad et al., 2000).

*3.4 Characterization of compounds **1-3***

The characterization of compound **1** is given below.

3.4.1 (-)-(2*R*,3*R*)-uncariechin (**1**)

Pale yellow amorphous solid, mp 249 – 250 ºC. $[\alpha]D^{20}$ -312.42º (MeOH, *c*0.015); MS *m/z* = 325.0724 $[M-H]^+$, (calcd: $[M]^+$ 326.0719)) $C_{18}H_{14}O_6$; UV (MeOH) λ_{max} nm: 276, 248, 216; IR (KBr) υ_{max} cm^{-1}: 3435, 3239, 1685, 1620, 1602, 1522, 1457; ^{1}H NMR (Acetone-*D*, 300MHz) δ ppm : 8.06 (1H, *d*, *J* = 9.6 Hz, H-4”), 7.44 (2H, *d*, *J* = 8.4 Hz, H-2’, H-6’), 6.88 (2H, *d*, *J* = 8.7 Hz, H-3’, H-5’), 6.42 (1H, *s*, H-8), 6.07 (1H, *d*, *J* = 9.6 Hz, H-3’’), 5.17 (1H, *s*, H-), 4.38 (1H, *m*, H-3), 3.00 (3H, *br s*, 4’-OH, 5-OH, 3-OH), 2.99 (1H, *dd*, *J* = 3.0, 15 Hz, H-4α), 2.94 (1H, *dd*, *J* =3.0, 15 Hz, H-4β);^{13}C NMR (Acetone-*D*, 75MHz) δ ppm: 160.55 (C-2’’), 160.15 (C-9), 157.07 (C-4’), 154.81 (C-5), 152.16 (C-7), 138.64 (C-4’’), 129.70 (C-1’),

128.17 (C-2'), 128.17 (C-6'), 114.78 (C-3'), 114.78 (C-5'), 109.71 (C-3''), 103.89 (C-10), 102.08 (C-6), 94.45 (C-8), 79.46 (C-2), 64.97 (C-3), 28.43 (C-4).

3.5 Computational method

TDDFT calculations were carried out at 298K in the gas phase with Gaussian 09 (Frisch et al., 2010). For the conformational search as well as the ECD, the absolute configuration of **1** (2*R*,3*R*) was chosen. The conformational search and geometry optimization were carried out at the molecular mechanics level of theory employing MMFF force field incorporated in Spartan08 (Wavefunction, Irvine, CA) software package. The conformers were selected and further geometry optimized at the modest B3LYP/6-31G (d,f) level of theory and TDDFT at the B3LYP/6-31(d,f) level of the theory basis set employed to simulate the ECD spectrum. The predicted wavelengths were used without any scaling. The adequacy of B3LYP/6-31(d,f) to optimize the geometry and to calculate the ECD spectra of flavan-3-ols similar to compound **1** have been demonstrated previously (Ding et al., 2010).

3.6 Cell line and culture conditions

The human neuroblastoma cell line, SH-SY5Y were acquired from Dr. Carol Sanfeliu (Department of Pharmacology and Toxicology, Institute of Biological Research, Barcelona, Spain). The SH-SY5Y cell line was originally established from a bone marrow biopsy of a neuroblastoma patient and is a third successive subclone of the parent cell lines SK-N-SH (Hana et al., 2010). Original studies by Pahlman et al. (1984) reported that SH-SY5Y possess neuron-like properties, including neurite outgrowth, and morphological changes, and have been extensively used as an *in vitro* model for CNS. Neuroblastoma (SH-SY5Y) cells were adapted to grow in 1:1 of Minimum Essential Medium Eagle (EMEM) (Sigma, USA). Nutrient mixture F12-Ham (Sigma, USA) supplemented with 1% non-essential amino acids (PAA Laboratories Gmbh, Austria), 1% L-glutamine (Sigma, USA), 1% 50 µg/ml gentamicin (PAA Laboratories Gmbh, Austria), and 10% fetal bovine serum (PAA Laboratories Gmbh, Austria). The cells were maintained in 5% CO_2 incubator (Contherm Scientific Ltd, New Zealand) at 37°C with 95% humidity.

3.7 Differentiation of cell line by retinoic acid

The SH-SY5Y cells were allowed to achieve 80-100% confluency in a tissue culture flask with an estimated number of 10^6 cell/ml. Approximately 2 x 10^4 cells/ml were seeded onto a 96-well plate and incubated for 24 hours. The cell were then induced to differentiate to become neuronal-phenotypic cells by adding 10 μM retinoic acid (RA) [Sigma, USA] and further incubated in a humidified atmosphere containing CO_2 at 37°C. The media was changed after three days with fresh RA and cells were ready to be used on the 6th day.

3.8 Neurotoxic and neuroprotective assay

Neurotoxicity tests were performed by incubating cultured cells ($1x10^4$cells/ml) with ranges of test compound concentrations (1nM – 1mM) overnight in a humidified atmosphere containing 5% CO_2 at 37 ºC. The results were assessed by the MTS assay on the next day. For the neuroprotection assay, the cultures were incubated with a serial dilution of compounds at final concentrations ranging from 1 nM to 1mM for 2 h. Cells were subsequently exposed to 230 μM hydrogen peroxide (H_2O_2, 30%, MERCK, Germany, which caused 52% of cell viability) before being treated with test compounds. The cultures were further incubated for 24 h, and then cell viability was again determined by the MTS assay. Results were representative of at least three independent experiments, and expressed as percentage of the value observed without any treatment (negative control). Cells treated with H_2O_2 served as a positive control.

3.9 Statistical analysis

Each experiment was carried out at least in triplicate. Data were reported as mean ± standard error (SE) of six replicate readings. The significance of differences among different groups was determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test using GraphPad PRISM Version 5.0 whereby positive significance was indicated as asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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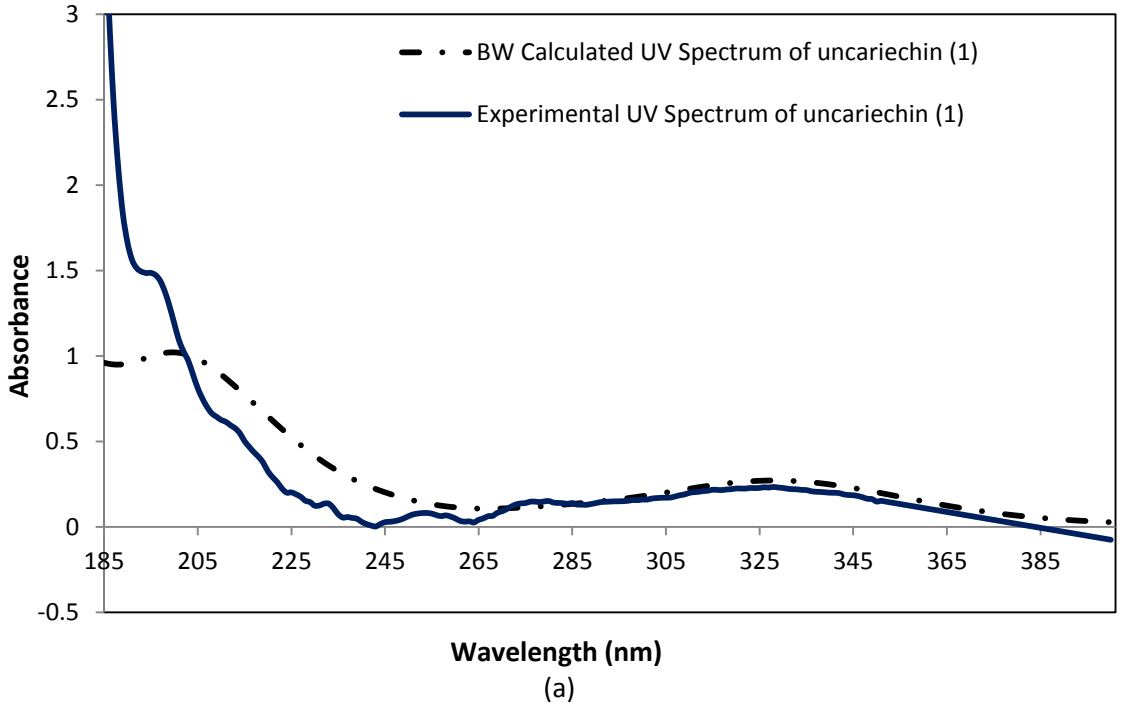
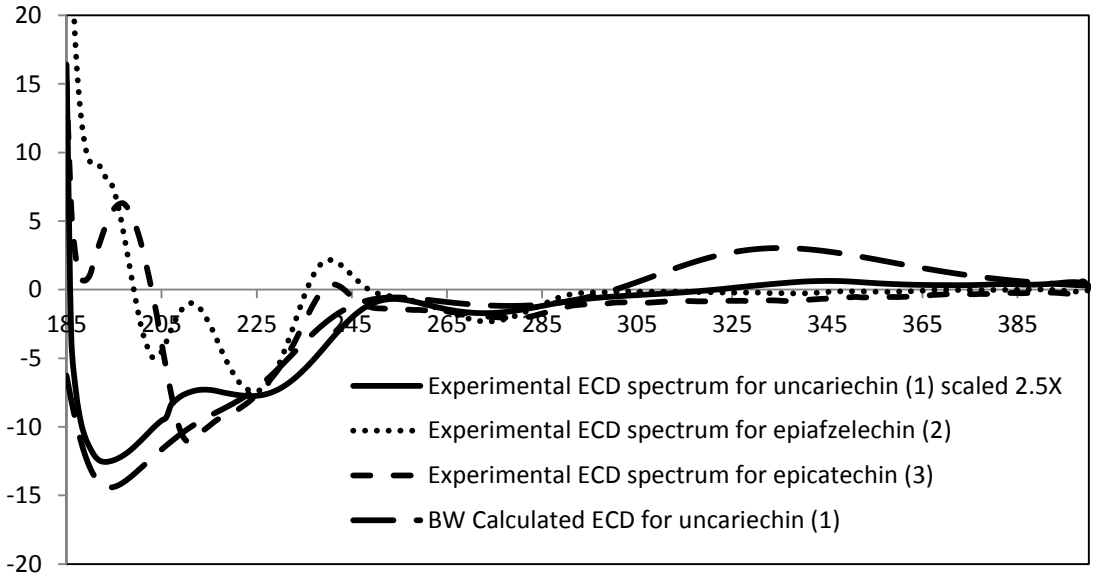


(a)



(b)

Figure 1: (a) Boltzmann weighted (BW) Calculated UV and Experimental UV spectra for compound 1 (b) Comparison of Boltzmann weighted Calculated ECD and Experimental ECD spectra for compounds 1, 2 and 3.

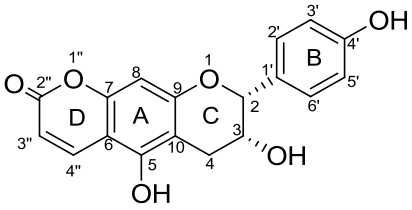


Figure 2: Structure of Compound **1**

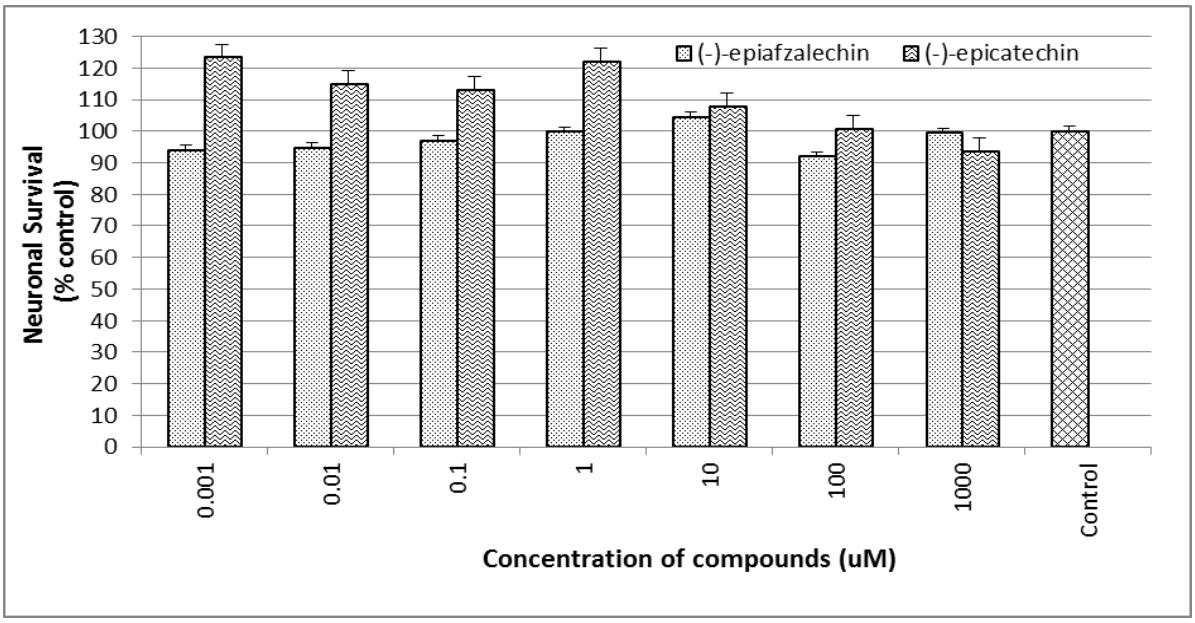


Figure 3: Neurotoxic properties of (-)-*epi*afzalechin and (-)-*epi*catechin against differentiated human neuroblastoma SH-SY5Y cell viability, assessed by MTS assay after 24 hours exposure at 37°C. Data is presented as mean ± SEM (n=6).

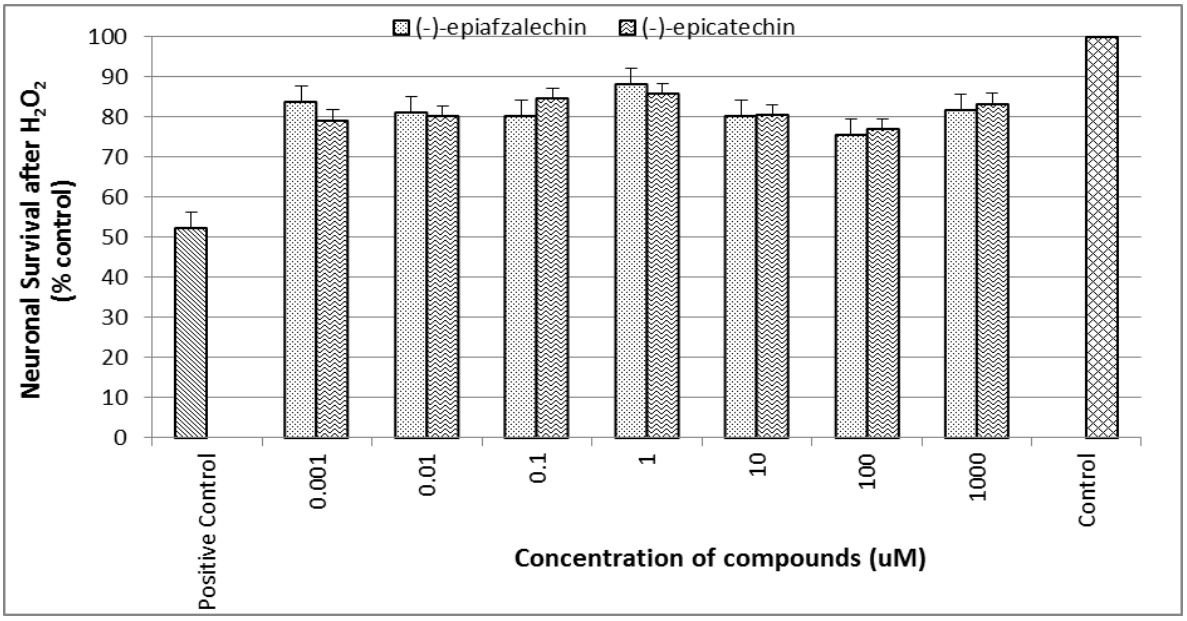


Figure 4: Neuroprotective properties of (-)-*epi*afzalechin and (-)-*epi*catechin against H_2O_2-induced neurotoxicity on differentiated human neuroblastoma SH-SY5Y as assessed by MTS assay after 24 hours of incubation at 37°C 5% CO_2. Data is presented as mean ± SEM (n=6)