Neuroprotective activity of natural products isolated from *Senecio graciliflorus* DC against corticosterone-induced impairment in SH-SY5Y cells

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Abstract

*Senecio graciliflorus* DC root extract was studied for secondary metabolite composition following the bioactivity-guided isolation technique. The ethyl acetate extract of *Senecio graciliflorus* root yielded nine chemical constituents: 3,4-di-tert-butyl toluene, stigmasterol, β-sitosterol, 2β-(angeloyloxy)furanoeremophilane, gallic acid, 2β-[(Z)-2-hydroxymethylbut-2-enoyl]oxy]furanoeremophilane, 1-hydroxypentan-2-yl-4-methylbenzoate, sarcinic acid, and sitosterol 3-O-β-D-glucopyranoside. The structures of the chemical constituents were elucidated on the basis of spectral data analysis in the light of literature. All the compounds are being reported for the first time from this plant. The isolated constituents were screened for neuroprotective effects against corticosterone-induced impairment in neuroblastoma cell lines (SH-SY5Y cells). The viability of SH-SY5S cells was determined using MTT assay. Among various isolated compounds, three natural products (sarcinic acid, gallic acid, and β-sitosterol) displayed robust neurotropic activity. The compounds increased neuronal cell survival in differentiated neuroblastoma cells (SH-SY5Y) from high-dose corticosterone (400 µM)–induced cell death. All the three constituents showed maximum AKT/ERK pathway activation at 20 µM concentration. The studies are aimed to explore small molecules for treating neurodegeneration underlying various neurological disorders to restore neuronal cell plasticity.

Keywords *Senecio* · Sterol · Sarcinic acid · Neuroprotection · Corticosterone

Introduction

*Senecio* (tribe Senecioneae) is the largest genus of family *Asteraceae* (Compositae) consisting of more than 1500 species with worldwide distribution. The genus has been extensively studied for secondary metabolite composition (Loizzo et al. 2004). Pyrrolizidine alkaloids (PAs), eremophilanolides, and cacalolides are the characteristic classes of compounds contained in the genus (Burgueno-Tapia et al. 2004). *Senecio species* not only enjoy positive impact but also have a negative impact in many agricultural countries, because such plants cause more deaths to livestock than any other poisonous plants. Their toxicity concerns are mainly attributed to their content of pyrrolizidine alkaloids and some furanoeremophilanes (Burgueno-Tapia and Joseph-Nathan 2003). *Senecio species* not only enjoy positive impact but also have a negative impact in many agricultural countries, because such plants cause more deaths to livestock than any other poisonous plants. Their toxicity concerns are mainly attributed to their content of pyrrolizidine alkaloids and some furanoeremophilanes (Burgueno-Tapia and Joseph-Nathan 2003).
flavonoids, diterpenes, triterpenes, and sesquiterpenes (F. Bohlmann F, Zdero C, Jakupovic J, Grenz M, Castro V, Kino RM, Robinson H, Vincent LPD. 1986; Ahmed et al. 1991; Tan et al. 2010; Cheng et al. 1992; Rucker et al. 1999; Morf 2002). Macrocyclic senecionine-type PAs constitute an important group of naturally occurring N-containing compounds. These compounds are pharmacologically potent and characteristic secondary metabolites for most species of the genus Senecio which cause hepatotoxicity to animals (Suau et al. 2002). Senecio species possess antibacterial and antifungal activities (Loizzo et al. 2004). A thorough biological study of the genus Senecio has revealed that the extracts as well as compounds of the genus possess hepatotoxic, teratogenic, or carcinogenic effects; antifeedant activity, antimitotic effect, and inhibition of angiotensinogen-converting enzyme (ACE); and insecticidal and neurotoxic activity (Dong-Liang et al. 1992; Steenkamp et al. 2001; Reina et al. 2001, 2002; Tundis et al. 2005; Xu et al. 2003).

A number of natural products show neuroprotective activity, e.g. resveratrol, salidroside, protopanaxadiol (PPD), and betulin. Resveratrol possesses a protective effect on corticosterone-induced neurotoxicity in PC12 cells while PPD exerts a protective effect on PC12 cells against glutamate-induced mitochondrial dysfunction (Zhang y, He Y, Deng N, Chen Y, Huang J, Xie W, 2019; Bak et al. 2016). Salidroside reduces amyloid-β (Aβ) levels and Aβ deposition in brain. The neuroprotective effect of salidroside occurs through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Zhang et al. 2016).

Senecio graciliflorus DC grows in the alpine forests and meadows of Kashmir valley. It is locally known as “Baghghu”. The water extract of the leaves is a folk medicine for the treatment of skin rashes and eruptions (Koul 1997; Joshi et al. 2013). Earlier we have reported the essential oil analysis of different parts of S. graciliflorus DC using GC-FID and GC–MS. The essential oil contains α-pinene, cis-ocimene, 1,2,3-trimethylcyclohexane, and β-pinenes as the major constituents (Lone et al. 2014).

However, to the best of our knowledge, there are no reports about the secondary metabolites and neuroprotective effects of S. graciliflorus DC and its constituents. So, we have carried out bioactivity-guided isolation of natural products from S. graciliflorus and investigated the isolated components for neuroprotection against corticosterone-induced neurotoxicity using SH-SY5Y cells.

**Experimental section**

**Plant material and chemicals**

*Senecio graciliflorus* DC was collected from the high-altitude areas of the Gulmarg region of Kashmir valley in the month of September 2017. The plant was authenticated by Prof. A. R. Naqshi (ex professor), Department of Botany, University of Kashmir. Voucher specimen bearing no. IIIMH-SP-1001 was deposited in the herbarium of the institute, IIM Srinagar. Corticosterone was purchased from Sigma-Aldrich. Organic solvents and silica gel (60–120) used were purchased from Merck. Media along with supplements were procured from Gibco. Natural molecules were dissolved in cell culture–grade DMSO (Sigma) and stored at –20 °C.

**Preparation of extract**

One hundred grams of shade-dried shoot and root parts was first processed using ethyl acetate as the extractive solvent to furnish shoot and root extracts for screening. After preliminary screening of shoot and root extracts for neuroprotection, only the root part was selected for phytochemical and bioevaluation studies. The root part of the plant was processed separately, chopped, oven dried at 40 °C, and ground to fine powder. The powdered roots (2.0 kg) were extracted sequentially by maceration with ethyl acetate using the cold extraction method at room temperature. The plant material was dipped in 6 l of ethyl acetate for 24 h prior to first wash of extraction. Three successive washings of the plant material were carried out, after 24 h each, to get the maximum constituents extracted. The solvent was removed under vacuo on a rotary evaporator to afford crude ethyl acetate extract of the root part (80.0 g) (Lone et al. 2013).

**Analytical techniques**

NMR spectra were obtained on 400-MHz Bruker spectrometer in deuterated chloroform (CDCl3) and deuterated methanol (MeOD) with tetramethylsilane (TMS) as the internal standard. Mass analysis was carried out on an LCMS-8030 mass spectrometer (Shimadzu Corporation, Kyoto, Japan). All the compounds were analysed in full-scan mode with nitrogen serving as an interface gas. Detection was done in ESI mode at a probe voltage of 180.0 V and a probe temperature of 400 °C. Column chromatography was carried on normal silica gel (60–120 mesh) (Merck grade), and pre-coated TLC plates with silica gel 60 F254 (Merck, 0.25 mm) were used for monitoring the column chromatography (Lone et al. 2013).

**SH-SY5Y cell culture**

Human neuroblastoma cell lines were propagated in DMEM-F12 media supplemented with 10% FBS, penicillin, and
streptomycin in CO2 atmosphere in a humidified incubator. Cells were subjected to differentiation using retinoic acid. Cells were collected and subjected to digestion in 0.05% trypsin–EDTA solution. Experiments were performed with cells within 12–18 passages (Constantinescu et al. 2007).

**Cell survival analysis**

Cells were incubated in 96-well plates (200 µl/well) with a seeding density of 1 × 104 cells/ml. After treatment, 20 µl MTT dye (5 mg/ml) was added to each well and kept for 2 h. The formazan crystals formed were dissolved in cell culture–grade DMSO, and the values were measured using a plate reader at 570 nm (Tiong et al. 2010).

**Corticosterone-induced cell impairment model**

Cell-based toxicity assay was established to induce cell impairment in cells subjected to corticosterone (10 µl of 400 µmol/l) treatment; cell survival was mainly dependent upon addition of certain external neurotropic factors (DHF/molecules). MTT assay was used to determine percentage cell survival after 48 h of compound treatment (Chen et al. 2013).

**Statistical analysis**

The GraphPad Prism software (version 8.4.3.686) was used for statistical analysis followed by one-way ANOVA. Experiments were replicated in set of triplicates, and the results were assessed as mean of three independent experiments, and the significance was expressed at p < 0.01.

**Result and discussion**

**Isolation of chemical compounds**

The constituents from *S. graciliflorus* were isolated following bioactivity-guided isolation and purification methodology. Both the shoot and root extracts were first screened for cell viability against corticosterone-induced toxicity in a differentiated human neuroblastoma cell line (SH-SY5Y).

The extracts were screened for cell viability in both the presence and absence of corticosterone. In contrast to the shoot extract, the root extract showed either similar or slightly better cell viability than the control at two different concentrations (10 µM and 50 µM) (Fig. 1). So only root extract was undertaken for isolation and identification of active constituents. Thus, 60.0 g of the ethyl acetate extract of *S. graciliflorus* DC root was subjected to fractionation and purification of constituents using normal-phase column chromatography.

Four fractions Fr-1, Fr-2, Fr-3 and Fr-4 of root extract were obtained using hexane–EtOAc and methanol as eluent with increasing polarity (Flow chart 1). Purification of Fr-1 yielded two constituents C-01 and C-02. Similarly, C-03 and C-04 and C-05 and C-06 were obtained from Fr-2 and Fr-3 respectively. Repeated column chromatography of Fr-4 yielded C-07, C-08, and C-09 as pure isolates. The structures of isolated constituents were characterised based on spectral data analysis in light of the literature.

Thus, the isolated natural products were identified as 3,4-di-tert-butyl toluene (C-01), stigmasterol (C-02), 2β-\{[(Z)-2-hydroxyethylbut-2-enoyl]oxy\}furanoeremophilane (C-03), gallic acid (C-04), β-sitosterol (C-05), 2β-(angeloyloxy)furanoeremophilane (C-06), 1-hydroxyptan-2-yl-4-methylbenzoate (C-07), sarcinic acid (C-08), and sitosterol 3-O-β-d-glucopyranoside (C-09) (Fig. 2) (Constantinescu et al. 2007; Tiong et al. 2010; Chen et al. 2013; Hambley et al. 1990; Nayak et al. 2015; Bohmann and Ziesche 1980; Gnawali et al. 2013; Awad et al. 2000). All the compounds were isolated for the first time from *S. graciliflorus* DC.

Compound C-01 was obtained as an amorphous powder and exhibited a molecular ion peak at *m/z* 204.17. The 1HNMR showed a singlet at δ 7.34 and two doublets at δ 7.53 and 7.10 with a coupling constant equal to 8.8 Hz indicating the presence of a phenyl ring substituted at three carbons. In addition, three singlets at δ 1.27 (9H), 1.32 (9H) and 1.55 (3H) were also seen in the 1HNMR spectrum. The 13CNMR showed six aromatic carbons at δ 147.85, 147.31, 138.69, 138.69, 124.69, 124.20, and 119.33 and five carbon resonances at 35.10, 34.75, 31.66, 30.43, and 29. Eight carbon signals of two tert-butyls appear as four signals as the carbons are magnetically equivalent. The structure of C-01 was thus determined as 3,4-di-tert-butyl toluene on the basis of the above 1HNMR, 13CNMR, and MS data in light of the literature (Hambley et al. 1990).

Compound C-02 was obtained as colourless needles and exhibited a molecular ion peak at *m/z* 412.37 (calculated for C29H48O, 412.37). The IR spectrum showed the hydroxyl bands at 3418 and 1640 cm−1 assignable to a secondary hydroxy functional group and a trisubstituted double bond. The 1HNMR displayed resonance signals at δ 7.53 and 7.10 with a coupling constant equal to 8.8 Hz indicating the presence of a phenyl ring substituted at three carbons. In addition, three singlets at δ 1.27 (9H), 1.32 (9H) and 1.55 (3H) were also seen in the 1HNMR spectrum. The 13CNMR showed six aromatic carbons at δ 147.85, 147.31, 138.69, 124.69, 124.20, and 119.33 and five carbon resonances at 35.10, 34.75, 31.66, 30.43, and 29. Eight carbon signals of two tert-butyls appear as four signals as the carbons are magnetically equivalent. The structure of C-01 was thus determined as 3,4-di-tert-butyl toluene on the basis of the above 1HNMR, 13CNMR, and MS data in light of the literature (Hambley et al. 1990).

Compound C-02 was obtained as colourless needles and exhibited a molecular ion peak at *m/z* 412.37 (calculated for C29H48O, 412.37). The IR spectrum showed the hydroxyl bands at 3418 and 1640 cm−1 assignable to a secondary hydroxy functional group and a trisubstituted double bond. The 1HNMR displayed resonance signals due to two quaternary methyl groups at δ 0.73 (3H, s, H-18) and 0.90 (3H, s, H-19) and three secondary methyl groups at δ 0.93 (3H, d, J = 6.5 Hz, H-21) and δ 0.86 (6H, d, J = 5.0 Hz, H-26) besides a resonance signal due to a primary methyl group at δ 0.71 (3H, m, H-29). Additional resonances in the 1HNMR spectra at δ 5.05 and δ 4.86 (1H each, dd, J = 15.5, 8.2 Hz, H-22, 23) confirmed a disubstituted double bond. Comparison of the physical characteristics and spectral data of C-02 with that reported in literature (Nayak et al. 2015) confirmed the structure as β-stigmasteryl.
Compound 03 was isolated as a colourless liquid with a molecular ion peak at \( m/z = 332.22 \). \(^{13}\)CNMR revealed the presence of twenty carbon signals assignable to four methyls, five methylenes, five methines, and six quaternary carbon resonances. \(^1\)HNMR shows two singlet protons in the aromatic region at \( \delta 7.69 \) and \( \delta 7.49 \), two alcoholic protons resonating at \( \delta 4.28 \), and a proton resonance at \( \delta 4.06 \) attached to an oxygenated carbon in addition to 24 protons resonating in the aliphatic region (\( \delta 0.86 \) to 2.34). \(^1\)HNMR, \(^{13}\)CNMR, and MS of C-03 is in complete agreement with that reported in literature (Bohlmann and Ziesche 1980). Thus, C-03 was identified as 2β-([(Z)-2-hydroxymethylbut-2-enoyl]oxy)furanoeremophilane.

Compound C-04 was isolated as a white solid having molecular mass at \( m/z = 170.12 \) for [M]\(^+\) corresponding to the molecular formula C\(_7\)H\(_8\)O\(_5\). IR spectra displayed

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Fig. 1 Screening of compounds using human neuroblastoma cell lines. a Survival analysis of differentiated human neuroblastoma cell line (SH-SY5Y) incubated with extracts in presence and absence of corticosterone: control (CON), corticosterone (CORT), shoot extract (LV), shoot extract and corticosterone (LV/C), root extract (RT), and root extract and corticosterone (RT/C). b Screening of molecules isolated from root extract for cell survival activity. c Cell viability after treatment of cells with increased concentration of C-04, C-05, and C-08.
absorptions at 3539 cm\(^{-1}\) due to hydroxyl groups, at 1701 cm\(^{-1}\) due to a carbonyl group, and at 1600, 1521, and 1497 cm\(^{-1}\) (aromatic moiety). The \(^1\)HNMR spectrum of compound C-04 displayed just one resonance signal, a characteristic resonance signal at \(\delta 7.0\) ppm assigned to two aromatic protons (H-2 and H-6). In its \(^{13}\)CNMR spectrum, compound C-04 displayed prominent resonance signals at \(\delta C 170.53\), assignable to the carboxylic acid group. Besides, the compound displayed signals for quaternary carbons at 139.71 assigned to the chemically equivalent C-3 and C-5 positions and at 146.48 corresponding to the C-4 position of the aromatic moiety. The single carbon resonance at 110 ppm in the distortionless enhancement by polarisation transfer (DEPT) spectrum depicted just one resonance signal, C-04 showed the presence of 29 signals. \(^1\)HNMR displayed prominent resonances at \(\delta 7.0\) ppm assigned to two aromatic protons (H-2 and H-6). In its \(^{13}\)CNMR spectrum, compound C-04 displayed prominent resonance signals at \(\delta C 170.53\), assignable to the carboxylic acid group. Besides, the compound displayed signals for quaternary carbons at 139.71 assigned to the chemically equivalent C-3 and C-5 positions and at 146.48 corresponding to the C-4 position of the aromatic moiety. The single carbon resonance at 110 ppm in the distortionless enhancement by polarisation transfer (DEPT) spectrum depicted just one resonance signal, C-04 showed the presence of 29 signals. 

Compound C-05 was isolated as a white solid powder with atmospheric pressure chemical ionization (APCI) mass at \(m/z\) 414.38 (M\(^+\)) melting point 136–140 °C. The MS showed two important fragments at \(m/z\) 383.36 (M\(^+\)-CH\(_3\)-OH) and \(m/z\) 255.21 (M\(^+\)-H\(_2\)O-alkyl side chain). Analysis of \(^1\)HNMR and \(^{13}\)CNMR of compound C-05 showed the presence of 29 signals. \(^1\)HNMR displayed resonance signals due to two quaternary methyl groups at \(\delta 0.69\) (3H, s, C-18) and 0.97 (3H, s, C-19), three secondary methylene groups at \(\delta 0.90\) (3H, d, \(J = 6.5\) Hz, C-21) and 0.87 (6H, d, \(J = 5.0\) Hz, C-26, H-27) and one signal due to a primary methyl group at \(\delta 0.80\) (3H, t, C-29). The \(^1\)HNMR displayed a resonance signal at \(\delta 5.34\) (1H, d, \(J = 3.1\) Hz, H-6) due to a trisubstituted double bond. All the data is in conformation with the previous literature reports identifying C-05 as \(\beta\)-sitosterol (Awad et al. 2000).

Compound 06 was isolated as a colourless liquid having mass at \(m/z\) = 316.20 corresponding to molecular formula C\(_{20}\)H\(_{22}\)O\(_{3}\). Close analysis of \(^{13}\)CNMR revealed the presence of twenty carbon signals assignable to five methyls, four methylenes, five methines, and six quaternary carbon resonances. Mass fragments at \(m/z\) 216 confirmed the presence of an angeloyloxy group, which was also supported by the presence of carbon signals corresponding to two vinylic methyls and a \(\beta\)-unsaturated ester. A mass fragment at \(m/z\) = 83 corresponds to the fragment C\(_6\)H\(_7\)CO which constitutes the furan moiety indicating the molecule to have a furanoeremophilane framework. Further analysis of spectral data along with the literature reports confirmed the molecule C-06 to be 2\(\beta\)-((angeloyloxy)furanoeremophilane (Bohlmann and Ziesche 1980).

C-07 was isolated as a viscous compound with \(m/z\) at 223.12(M + 1). The \(^1\)HNMR showed two doublets at \(\delta 7.68\) (2H) and at \(\delta 7.48\) (2H) indicating the presence of a 1,4-substituted aromatic ring. The presence of a singlet at \(\delta 1.73\) (3H) indicates that a methyl is attached to the phenyl ring. A triplet at \(\delta 4.27\) and a corresponding carbon resonance at \(\delta 65.66\) represent –CH\(_2\)OH. A carbonyl carbon resonates at \(\delta 167.82\). In addition, the \(^1\)HNMR shows six more protons at \(\delta 1.45–1.23\) (4H) and \(\delta 0.96\) (3H). Comparison of the spectral data (NMR and MS) with that reported earlier resulted in the identification of C-07 as 1-hydroxypentan-2-yl-4-methylbenzoate (Yeon et al. 2002).

Compound C-08 was isolated as yellow-coloured oil with APCI mass at \(m/z\) 116.12 corresponding to the molecular formula C\(_6\)H\(_8\)O\(_3\). The \(^{13}\)CNMR spectra of C-08 displayed five signals. The DEPT NMR revealed a vinylic methyl with the corresponding signals in \(^1\)HNMR at \(\delta 2.10\) ppm (d, \(J = 4\) Hz, 3H). A downfield doublet at \(\delta 6.25–6.62\) corresponding to one proton along with the carbon signals at 172, 144, and 131 ppm suggested an \(\alpha\)-substituted enone with the carbonyl moiety in acid form. The presence of a carbon signal at \(\delta 65\) in DEPT NMR suggested the occurrence of a primary alcoholic group. All these observations
along with the previous literature data confirmed C-08 to be sarcinic acid (Shuzo and Kunihiko 1960).

Compound C-09 was isolated as yellow-coloured amorphous powder having m.p. 272–274 °C from the ethyl acetate extract. The $^1$HNMR spectrum showed the olefinic protons as a broad singlet at $\delta$ 5.09 indicative of the presence of a $\mathrm{C=CH}_2$ in the ring system. A number of multiplet signals between $\delta$ 1.12 and 2.14 were informative of different methylene and methine protons in the structure of compound C-09. The resonances at $\delta$ 3.13–4.39 in the $^1$HNMR indicated the sugar moiety. The spectrum showed a multiplet at $\delta$ 3.51 indicative of an oxymethine proton (H-3). The $^{13}$CNMR spectrum revealed the presence of 35 carbons suggestive of a steroidal compound (29 signals) with six signals corresponding to a sugar moiety. The signals at $\delta$ 141.01, 42.41, and 36.17 were assigned to three quaternary carbons, and a signal at $\delta$ 71.85 was for oxymethine carbon. All the above data with the literature reports confirmed compound C-09 to be sitosterol-3-$\beta$-d-glycoside (Jares et al. 1990).

**Natural compounds as potential pro-survival entities against neural cells (SH-SY5Y)**

To search for natural molecules with potential cell survival activity, cell line–based assay was employed. Among the library of compounds screened, only three compounds showed significant cell survival activities from the root part of plant (Fig. 1). C-04, C-05, and C-08 inhibited the cell impairment caused by corticosterone in differentiated neuroblastoma cell line (SH-SY5Y). Effective dosage of 400 µM resulted in 40–60% reduction of cell viability in differentiated neuroblastoma cell lines, which is in agreement with previously published reports (Gite et al. 2019; Gao et al. 2015). In rats and gerbils, high levels of corticosterone cause impairment in hippocampal neurons (Yusim et al. 2000). Furthermore, corticosterone when employed at a higher concentration, i.e. $>250$ nm, adds to neuronal cell death in vitro and in vivo (Abrahám et al. 2006). To determine LD50 values of corticosterone, a dose–response curve was created by incubating human neuroblastoma cell cultures (SH-SY5Y) with corticosterone in a concentration-dependent manner for 24 h followed by MTT assay for assessing the viability of cells. Cells incubated with doses $>100$ µM showed reduced cell viability; those incubated with 400 µM showed only 45% viability, considering it as standard value for performing experiments.

**Neuroprotective activity of selected hits against corticosterone-induced cell toxicity**

Human neuroblastoma cell lines were pre-incubated with positive hits (24 h), later subjected to high-dose corticosterone (400 µM) treatment again for 24 h (Fig. 3). Corticosterone induces impairment in differentiated cell lines showing obvious injury, reversed by the selected hits, thereby increasing cell survival (Liu et al. 2015). Initially, differentiated human neuroblastoma cell lines were incubated with root and shoot extracts in the presence and absence of corticosterone for 48 h. Though most of the constituents showed positive effects on...
cell viability, three constituents (C-04, C-05, and C-08) were capable of restoring cell survival (Fig. 1).

MTT assay was used for evaluating the cytotoxicity of molecules on human neuroblastoma cells where the cell viabilities are more than 100%, indicating compounds may be of proliferative nature, potentially dividing cells after treatment as the cells subjected to corticosterone-induced impairment also showed increased viability when compared to the negative control (Kim et al. 2020; Han et al. 2014).

Protective effects of natural products (C-04, C-05, and C-08) against corticosterone-induced cytotoxicity in human neuroblastoma cell lines (SH-SY5Y)

The effect of natural isolates (C-04, C-05, and C-08) on the viability of human neuroblastoma cell line SH-SY5Y cells was studied in the presence and absence of corticosterone. Previous studies found that PEG-PEI/siROCK2 protects against amyloid-beta-induced neurotoxicity (Gao et al. 2015). The viability of SHSY5Y cells was determined using MTT assay. Human neuroblastoma cell lines were pre-incubated with the three natural products C-04, C-05, and C-08, which showed the best cell viability in preliminary screening, for 24 h and later subjected to corticosterone treatment for another 24 h.

As expected, corticosterone treatment at 400 µM concentration induced cell impairment, which resulted in decreased cell viability. Similar to DHF (5 nM) treatment, the cell viability remained unaffected, compared to the control, when the cells were treated with C-04, C-05, and C-08 only at 20 µM concentration. On the other hand, the cell viability increased after the treatment of the cells with 20 µM solution of C-04, C-05, or C-08 for 24 h prior to corticosterone treatment (Fig. 3). These results clearly indicate that the isolated constituents (C-04, C-05, and C-08) from S. graciliflorus show neuroprotective effects on SH-SY5Y cell lines. Similar results have earlier been observed for resveratrol wherein resveratrol has shown a protective effect against corticosterone-induced neurotoxicity in PC12 cells (McCarty and DiNicolantonio 2017).

![Fig. 3](link) Protective effects of natural isolates (C-04, C-05, and C-08) on SH-SY5Y cells in corticosterone-induced impairment using MTT assay. Cells were subjected to corticosterone-induced toxicity for 24 h after pre-incubating with compounds (**p < 0.01)
In another experiment, SH-SY5S cells were treated only with different concentrations of C-04, C-05, and C-08 without the treatment of corticosterone. The treatment of SH-SY5S cells with C-04, C-05, and C-08 showed a dose-dependent response. The cell viability increased up to 31.25 µM concentration of all the three natural products C-04, C-0, and C-08 but decreased beyond this concentration and proved to be toxic at higher concentrations. Though lower concentrations of the positive compounds enhanced cell viability, their effects after a certain dose level provoke neuronal damage in differentiated neuroblastoma cell lines (Fig. 1c).

Screening of C-04, C-05, and C-08 for activation of various survival proteins

All the three natural isolates C-04, C-05, and C-08 which showed neuroprotection were screened for activation of survival proteins using differentiated neuroblastoma cells under standard optimised conditions of temperature and humidity. Earlier SH-SY5Y cells were subjected to differentiation 5 days prior to incubation with compounds in a concentration-dependent manner for 48 h. Multiple line experiments were performed to study the effect of positive hits (C-04, C-05, and C-08) on neuronal cell survival and activation of various survival pathways.

Earlier reports show that certain molecules prevent amyloid-beta-induced neurotoxicity and that the activation of the PI3K/Akt pathway is involved in neuroprotection. Since the AKT/PI3K signalling pathway plays an important role in promoting neuronal survival (Zhang y, He Y, Deng N, Chen Y, Huang J, Xie W, 2019; McCarty and DiNicolantonio 2017), the mechanism by which C-04, C-05, and C-08 protect SH-SY5Y cells from corticosterone-induced neurotoxicity was particularly explored by studying the effect of these constituents on AKT and ERK activation. AKT/PI3K pathway activation has been shown to prevent amyloid-β-induced neuronal neurotoxicity (Tiwari et al. 2015).

We examined the phosphorylation of AKT and ERK. Though all the three isolates were studied for phosphorylation of AKT/ERK, only C-08 showed predominant phosphorylation of AKT/ERK in SH-SY5Y neuroblastoma cells (Fig. 4). AKT/ERK expressions were also studied after the treatment of cells with different doses of C-08, and the results showed that the maximum AKT/ERK pathway activation occurred at 20 µM concentration (Figs. 4 and 5).

C-08 induces AKT and ERK activation in differentiated neuroblastoma cells in time-dependent manner

In order to study whether the neurotropic activity is time dependent, we determined the phosphorylation reaction at different time intervals after differentiating SH-SY5Y cells for a period of 5 days and later incubated with C-08 at optimum conditions of humidity and temperature. C-08 activated the survival pathway (p-ERK and p-AKT at 20 µM) in a time-dependent manner (Fig. 6).
Conclusion

In conclusion, *S. graciliflorus* DC was studied for both its secondary metabolite composition and neuroprotection. The secondary metabolite composition of the roots of *S. graciliflorus* DC yielded nine chemical constituents, which were identified as 3,4-di-tert-butyl toluene, stigmasterol, β-sitosterol, gallic acid, 2β-(angelyoxy)
furanooeremophilane, 2β-[[Z]-2-hydroxymethylbut-2-enoyl]oxy]furanooeremophilane, sarcinic acid, sitosterol 3-O-β-D-glucopyranoside, and 1-hydroxypentan-2-yl-4-methylbenzoate. All the compounds are being reported for the first time from *S. graciliflorus* DC. The *in vitro* screening of the isolated constituents for neuroprotective effects against corticosterone-induced impairment resulted in the identification of three natural products (gallic acid, sarcinic acid, and β-sitosterol) with promising neuroprotective activity. All the three positive constituents enhanced cell survival/cell viability in corticosterone-induced toxicity in SHS5YS cells through AKT/ERK pathway activation in a concentration- and time-dependent manner.

**Supplementary Information** The online version contains supplementary material at https://doi.org/10.1007/s00210-021-02136-9.

**Author contribution** Khursheed Ahmad Bhat and Fayaz A. Malik conceived and designed the research work, supervised the experimental work, interpreted the experimental data, and participated in writing the manuscript. Salman Jameel and Loveleena Kaur did the experimental work and participated in writing the manuscript. Showkat Ahmad Bhat contributed to the isolation of pure natural products. All authors approved the final version of the article. The authors declare that all data were generated in-house and that no paper mill was used.

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**Data availability** The research data is available as a supplementary file.

**Declarations**

**Ethical approval** The work was reviewed and approved by institutional ethics and publication committee. The committee has assigned CSIR-IIM/IPR/00291 as the research paper number.

**Consent to participate** Not applicable.

**Consent for publication** All the authors agree to publish the research work.

**Competing interests** The authors declare no competing interests.

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