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In vitro protective effects of *Withania somnifera* (L.) Dunal root extract against hydrogen peroxide and β-amyloid$_{(1-42)}$ induced cytotoxicity in differentiated PC12 cells

Short title: Neuroprotective effects of *Withania somnifera* extract against H_2O_2 and β-amyloid

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ABSTRACT

Withania somnifera L. Dunal (Solanaceae), also known as 'ashwagandha' in Sanskrit and as 'Indian ginseng', is widely used in Ayurvedic medicine as a nerve tonic and memory enhancer, with anti-aging, anti-stress, immunomodulatory and antioxidant properties. There is a paucity of data on the potential neuroprotective effects of *W. somnifera* root, as traditionally used, against H_2O_2- and $A\beta_{(1-42)}$-induced cytotoxicity which are current targets for novel approaches to treat dementia, especially dementia of the Alzheimer's type (AD). In this study, an aqueous extract prepared from the dried roots of *W. somnifera* was assessed for potential protective effects against H_2O_2- and $A\beta_{(1-42)}$-aggregated fibril cytotoxicity by an MTT assay using a differentiated rat pheochromocytoma PC12 cell line. The results suggest that pre-treatments of differentiated PC12 cells with aqueous extracts of *W. somnifera* root significantly protect differentiated PC12 cells against both H_2O_2- and $A\beta_{(1-42)}$-induced cytotoxicity, in a concentration dependent manner. To investigate the compounds that could explain the observed effects, the *W. somnifera* extract was analysed by liquid chromatography-serial mass spectrometry and numerous withanolide derivatives, including withaferin A, were detected. These results demonstrate the neuroprotective properties of an aqueous extract of *W. somnifera* root and may provide some explanation for the putative ethnopharmacological uses of *W. somnifera* for cognitive and other neurodegenerative disorders that are associated with oxidative stress.

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Keywords: *Withania somnifera*; neuroprotection; hydrogen peroxide; amyloid; cytotoxicity; Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia, is a progressive neurodegenerative disease marked by a decline in cognitive functions, primarily memory loss and language deficit which are often accompanied by behavioural and psychological symptoms such as depression, stress, anxiety and mood disturbances. The pathophysiology of AD is complex and is characterized by neuronal degeneration (cholinergic neurons in particular), abnormal neurofibrillary tangles, toxic β-amyloid (Aβ) plaques and deficiencies of neurochemicals which are essential for neuronal transmission (Ros and Poirier, 2004). Aβ cytotoxicity to neuronal cells has been identified as one of the major features in AD pathology, but the exact mechanisms of the cascade of events leading to neurotoxicity still remain unclear (Fukuyama *et al*., 1994; Fagarasan and Efthimiopoulos, 1996; Yan *et al*., 1997; Canevari *et al.*, 2004). Some studies suggest that one pathway of Aβ induced cytotoxicity could be mediated by free radicals and oxidative stress (Hensley *et al*., 1994; Behl, 1994, 1997; Opazo *et al.*, 2000; Canevari *et al*., 2004). Evidence obtained using cultured cells, such as sympathetic neurons (Greenlund *et al*., 1995), has demonstrated that reactive oxygen species are produced during the early, but not the late phase of neuronal cell death, suggesting that their production serves as an early signal, rather than a toxic agent, to mediate apoptosis. It has also been demonstrated that Aβ increases concentrations of hydrogen peroxide (H_2O_2) in cells (Behl *et al.*, 1994). Hydrogen peroxide is one of the

typical inducers of apoptosis in neuronal cells (Whittemore *et al*., 1994; Satoh *et al.*, 1996).

Withania somnifera (L.) Dunal (Solanaceae) root (also known as 'ashwagandha' in Sanskrit and as 'Indian ginseng') is widely used in Ayurvedic medicine (Mishra *et al*., 2000; Houghton and Howes, 2005) as a nerve tonic and memory enhancer, with anti-aging, anti-stress, immunomodulatory and antioxidant properties (Ziauddin *et al.*, 1996; Archana and Namasivayan, 1999, Bhattacharya *et al.*, 2001; Jayaprakasam *et al*., 2003). Some studies have investigated a pharmacological basis to explain the reputed effects of *W. somnifera* on cognition, and to assess the therapeutic potential for the steroidal derivatives from this species, particularly the withanolides. Studies *in vitro* and *in vivo* have investigated the potential of *W. somnifera* to modulate cholinergic function and extracts of the root, and withanolides isolated from this species (withaferin A, 2,3-dihydrowithaferin A and 5β,6β-epoxy-4β-hydroxy-1-oxowitha-2,14,24-trienolide), inhibit acetylcholinesterase (Schliebs *et al*., 1997; Choudhary *et al*. 2004, 2005; Houghton *et al*. 2007; Vinutha *et al*. 2007).

Other studies show that extracts of *W. somnifera* root, and some of the withanolide derivatives in particular, promote dendrite formation in human neuroblastoma cells *in vitro* in a dose-dependent manner (Tohda *et al.*, 2000; Zhao *et al.*, 2002); withanolide A and withanosides IV and VI extend axons and dendrites, respectively, *in vitro* (Kuboyama *et al*., 2002), and withanolide A is considered to reconstruct neuronal networks *in vivo* (Kuboyama *et al*., 2005). In an animal model of dementia and spinal cord injury, withanolide A, withanoside IV, and withanoside VI restored presynapses and postsynapses (Tohda *et al.*, 2000, 2005). Other studies have investigated various *W. somnifera* extracts *in vivo* to explain the putative effects of *W. somnifera*, including

potential neuroprotection against various models of oxidative stress and cytotoxicity, and disease mechanisms have been explored, with varying results (Parihar and Hemnani, 2003; Sankar *et al.*, 2007; Bhatnagar *et al.*, 2009). Root extracts from this species have also been shown to significantly reduce the number of hippocampal degenerating cells in the brains of stressed rodents (Jain *et al.*, 2001) and were neuroprotective in animal models of Parkinson's disease (Ahmad *et al.*, 2005; Sankar *et al.*, 2007) and Huntington disease (Kumar and Kumar, 2009) . Although some *in vivo* studies using various animal models suggest *W. somnifera* extracts can improve antioxidant status and provide neuroprotection (Jain *et al.*, 2001; Parihar and Hemnani, 2003; Sankar *et al.*, 2007; Bhatnagar *et al.*, 2009), there is a paucity of data on the potential protective effects of *W. somnifera* root, as traditionally used, against H_2O_2- and $A\beta_{(1-42)}$-induced cytotoxicity which are current targets for novel approaches for the treatment of dementia, especially dementia of the Alzheimer's type (AD). The aim of this study was to investigate the potential neuroprotective effects of an aqueous extract of *W. somnifera* root against H_2O_2- and $A\beta_{(1-42)}$-induced toxicity under *in vitro* conditions using a differentiated PC12 cell line as a model of neuronal cells.

MATERIALS AND METHODS

Plant material. The roots of *W. somnifera* were purchased from a local herbalist in India and were verified and authenticated by Dr George Wake, School of Biology, Newcastle University, UK. A voucher specimen (SK-WS-01) is deposited in the Herbarium of the Medicinal Plant Research Group, Newcastle University, UK.

Extract preparation. The air dried roots were ground to powder consistency using an electric grinder. 1 g of powdered root was infused in freshly boiled de-ionised water (1:50 w/v) for 25 min. The infusion was left to cool to room temperature and centrifuged (12000 rpm, 15 min). The supernatant was re-centrifuged (12000 rpm, 10 min) and then freeze dried. The freeze dried aliquots were reconstituted in de-ionised water prior to assay**.**

Cell line and cell culture. Rat pheochromocytoma (PC12) cell line was a generous gift from the Medical School, Newcastle University. RPMI-1640, penicillin-streptomycin, foetal calf serum, glutamine and nerve growth factor (NGF) were purchased from Invitrogen (UK). $A\beta_{(1-42)}$ and trypan blue were purchased from Sigma (UK).
Cells were maintained in RPMI media supplemented with 10 % heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin-streptomycin in humidified 5 % CO_2 and 95 % air at 37 °C. All cells were cultured in culture flasks pre-coated with poly-D-lysine (0.1 mg/mL). Cells were differentiated for 2-3 days using 50 ng/mL NGF. Prior to experimental treatments, cells were microscopically examined to assess differentiation. Eighty percent or more of cells with neurite outgrowth extensions over 2–3-fold cell body size were considered to be differentiated PC12 cells (dPC12). The medium was changed every alternate day. Prior to confluence, cells were dislodged by mechanical scraping and split in 1:3 ratio. Before each experiment, cells were checked for viability using a trypan blue (0.5 %) dye exclusion method (Freshney, 2000). Cells were counted using a haemocytometer and the density was adjusted to $1x10^5$ cells/mL prior to plating in 96-well plates; cells in exponential growth phase were used.

Assay of H_2O_2-induced toxicity. Before treatment, cells were plated at an appropriate density ($1x10^4$ cells/100µL) in a 96-well plate and incubated for 24 h at 37 ^oC, so that the cells were acclimatized to the new environment. Cells were pre-incubated with *W. somnifera* root extract (concentration range: 0.097 – 50 µg/mL), prior to exposure to H_2O_2 (concentration range: 12.5 µM – 400 µM) from a freshly prepared 1000 µM stock solution. After 24 h the cell viability was determined by an MTT assay, as described below.

β-Amyloid$_{(1-42)}$ fibrils preparation. $A\beta_{(1-42)}$ peptide was stored at -20 ^oC until use at room temperature; lyophilized peptide was dissolved in distilled water at a concentration of 0.1 mg/mL, with thorough pipetting over a period of 2 min to clarify the solution. It was then incubated overnight at 37 ^oC with constant oscillation to form the fibrils used to induce toxicity in dPC12 cells.

Assay of β-amyloid$_{(1-42)}$-induced cytotoxicity. The *in vitro* toxicity of $A\beta_{(1-42)}$ to dPC12 cells was measured by incubating the cells for 24 h at 37 ^oC, with increasing concentrations (0.007 – 2 µg/mL) of aggregated $A\beta_{(1-42)}$ peptide in a 96-well plate. Cell viability was then assessed by measuring cellular redox activity with the MTT assay described below.

MTT assay. PC12 cell viability was determined by the MTT reduction assay. In brief, MTT, a tetrazolium salt, is cleaved to formazan by succinate dehydrogenase, an enzyme in the mitochondrial respiratory chain, by live cells. After pre-incubation of the dPC12 cells with the test extracts and toxicity inducers (H_2O_2 or Aβ) for 24 h, cells were

incubated with MTT (0.5 mg/mL) for 3 h. The dark blue formazan crystals formed in live cells were solubilised with dimethylsulfoxide and ethanol (1:1) and the absorbance was measured at 570 nm using a spectrophotometer (Molecular Device Spectramax Plus 384, equipped with Softmax Pro V5 software) (Mosmann, 1983).

Liquid chromatography-serial mass spectrometry. The freeze dried aqueous extract of *W. somnifera* root was analysed by liquid chromatography-serial mass spectrometry ($LC\text{-}MS^n$) using an HPLC system (Thermo Scientific 'Surveyor' autosampler, pumps and diode array detector) coupled to a 3D ion-trap mass spectrometer (Thermo Scientific 'LCQ Classic') via an electrospray source. Chromatography was achieved on a 150 mm x 4.6 mm, 5 µm C18 column (Phenomenex 'Luna C18(2)') using a 1 ml/min mobile phase gradient of 10 % to 100 % aqueous acetonitrile containing 0.1 % formic acid over 40 min. The flow to the ESI source was reduced to 200 µl/min by a splitter and the source was operated using a needle voltage of ±4.2 kV and desolvation nitrogen gas flows of 80 (sheath) and 20 (auxillary) units. The heated capillary temperature was 220 ºC and standard tuning voltages (obtained from rutin) were used. The instrument was operated using Xcalibur 2.0 software and components in the $LC\text{-}MS^n$ analyses were detected using MassFrontier 4.0 (both Thermo Scientific). Withaferin A (LGC Standards, UK) was also subjected to $LC\text{-}MS^n$ analysis using this method.

Statistical analyses. Results are expressed as the mean ± SEM. Student t-test was used to compare differences between test groups and the negative control (H_2O_2 or Aβ only) using Graphpad statistical software; $p<0.05$ were considered significant.

RESULTS

Effect of H_2O_2 on dPC12 cell viability after treatment for 24, 48 and 72 h

When dPC12 cells were incubated with different H_2O_2 concentrations (12.5 µM to 400 µM) at 24, 48, 72 h time intervals, a concentration-dependent cytotoxicity was observed (Fig. 1). The cell viability was decreased to 50 % at 200 µM H_2O_2 during 24 incubation, but less than 50% for 48 and 72 h incubation at 200 and 400 µM H_2O_2 ($p<0.001$). Thus, 200 µM H_2O_2 was selected as an appropriate concentration to induce toxicity, with an incubation time of 24 h, to assess the effects of the *W. somnifera* extract in further experiments.

Effect of *W. somnifera* extract on viability of dPC12 cells against H_2O_2-induced cytotoxicity

As shown in Fig. 2, the aqueous extract of *W. somnifera* significantly protected dPC12 cells from H_2O_2-induced toxicity when cells were pre-incubated with *W. somnifera* extract for 24 h prior to H_2O_2 (200µM) exposure. After H_2O_2 exposure for 24 h, 50 – 80 % cell viability was observed at extract concentrations from 6.11 µg/mL to 100 µg/mL, compared with the negative control (H_2O_2 alone), with 50 and 100 µg/mL extract concentrations showing the greatest improvement compared with the negative control ($p<0.001$). At the highest extract concentration (200 µg/mL) the cytoprotective effects were abolished, due, perhaps, to direct cytotoxic and antiproliferative properties of *W. somnifera* being expressed at high concentrations (Sabbaraju *et al*., 2006; Stan *et al*., 2008).

Effect of β-amyloid$_{(1-42)}$-peptide aggregates on dPC12 cells

. A concentration-dependent decrease of cell survival was observed after exposure to Aβ $_{(1-42)}$ over the concentration range of 0.007 to 2.0 µg/mL (Fig. 3). The cell survival was expressed as a percentage of the control measured in the absence of Aβ peptide. The cell viability was decreased to approximately 50 % at 0.5 µg/mL and no further decrease was observed by increasing the Aβ concentration above 0.5 µg/mL. Consequently, this concentration of Aβ was selected to induce cytotoxicity in further experiments to investigate the neuroprotective effect of *W. somnifera* extract against Aβ-induced toxicity.

Effect of *W. somnifera* extract on viability of dPC12 cells against β-amyloid$_{(1-42)}$-induced toxicity

The pre-treatment of *W. somnifera* extract (0.75 µg/mL – 100 µg/mL) for 24 h prior to Aβ incubation produced dose-dependent attenuation of Aβ-induced toxicity in dPC12 cells. Maximum viability (80 % of positive control) was observed at the highest concentration (100 µg/mL) tested (Fig. 4).

LC-MS^n analysis

Positive ion LC-MS^n analysis of the *W. somnifera* extract revealed numerous components (Fig. 5). Component **36** was identified as withaferin A against a standard and together with two other isomeric withanolides (**38** & **40**), these constituted the main withanolide aglycones in the extract. The majority of the other components, eluting earlier in the analysis, were assigned as derivatives of withanolides (Table 1). Among these were compounds clearly assignable as glycosides of withanolides (**10**, **21**, **22**, **25**,

27, **29**, **30**, **33 & 35**) since, following MS2 of the protonated or ammoniated molecules, they generated fragment ions resulting from cleavage of glycosidic bonds, including protonated aglycone ions at *m/z* values expected for reported withanolides. Furthermore, upon MS3 analysis, these protonated aglycone ions showed patterns of neutral losses similar to those observed following MS2 analysis of the free withanolides in the extract. More exact assignment of withanolides from *W. somnifera* was not determined due to the numerous isomeric forms of withanolides reported in the literature (CCD, 2010). Other components were also assigned as withanolide derivatives. Following MS2 of the protonated molecule they generated a complex array of product ions, but among these were those with expected *m/z* values for fragments corresponding to protonated withanolide aglycones. Furthermore, for components such as **14** that generated a product ion *m/z* 471 (the value expected for a fragment equivalent to protonated withaferin A and isomers), other product ions at *m/z* 299 and 281 were also observed; withaferin A also generated abundant product ions at these *m/z* values following MS/MS of the protonated molecule. Several of the components assigned as withanolide derivatives appeared to have odd-number molecular masses (i.e. suggesting they contained an odd number of nitrogen atoms), including component **12** that produced the largest chromatographic peak in the analysis. The major ion species generated by positive ESI of **12** (*m/z* 778) was assigned as $[M+H]^+$ on the basis of minor confirmatory ions at *m/z* 795 $[M + NH_4]^+$ and *m/z* 800 $[M + Na]^+$, and the generation of a major ion species at *m/z* 776 $[M - H]^-$ in a negative ion ESI analysis. The MS2 spectrum of protonated **12** showed minor product ions at *m/z* 471, 299 and 281, suggesting that the compound contained a withanolide moiety. The major product ion was at *m/z* 649 and MS3 analysis of this ion produced a spectrum that was almost

identical to the product ion spectrum of protonated **6** ($[M+H]^+$ = *m/z* 649). Although nitrogen-containing compounds (including withanamides that protect PC12 cells from Aβ (Jayaprakasam *et al*., 2009)) have been reported in the fruits of *W. somnifera*, none have the same molecular mass as **12** and do not display a potential aglycone product ion at *m/z* 471 (Jayaprakasam *et al*., 2004). An isomer of **12**, component **16**, also generated a major product ion at *m/z* 649, but serial MS analysis of this ion produced a different spectrum to that of **12**, in particular the spectrum suggested that the molecular mass of the aglycone was 452. Components **13** and **17** were likely hexosides of **12** (or its isomers) and a likely hexoside of **14** (or isomer) was also present (component **15**). Four components (**39**, **41**, **42** & **44**) in the LC-MS^n analysis were assigned as withanolide sulphates as their molecular masses showed an 80 Da increment over that of known withanolides. Other noted components in the extract were low molecular mass nitrogen containing compounds (**1**-**3**).

DISCUSSION

There is growing interest in naturally-derived bioactive compounds with potential neuroprotective properties against a range of neurodegenerative diseases. One area of current pharmacological focus is neuroprotective therapeutic strategies aimed at counteracting H_2O_2- and Aβ-induced neurotoxicity associated with AD pathophysiology. In this study, PC12 cells were used as an *in vitro* model to study the neuroprotective potential of *W. somnifera* root aqueous extract against H_2O_2- and Aβ-induced toxicity. PC12 cells were chosen as they acquire neuronal like projections when induced to differentiate with nerve growth factor. The differentiated PC12 cells are both morphologically and physiologically very similar to living neurons in the brain;

therefore the results obtained by neuroprotective studies with experimental test plant extracts will be more likely to represent the response when using differentiated, rather than with non-differentiated cells (Datki *et al*., 2003). To induce Aβ peptide toxicity, the peptide fragment with a length of 1–42 amino acid residues was used, as this fragment aggregates very rapidly and is responsible for inducing toxicity as compared with non-aggregated peptides. The toxic effects of $A\beta_{(1-42)}$ fragments have been well-documented in a number of studies (Behl *et al*., 1992; Michaelis *et al*., 1998; Limpeanchob *et al*., 2008).

The present study demonstrated that pre-treatments of differentiated PC12 cells with an aqueous extract of *W. somnifera* root significantly protected dPC12 cells against both H_2O_2- and Aβ-induced cytotoxicity, in a concentration dependent manner. As Aβ is known to increase free radical production and lipid peroxidation in PC12 cells, leading to apoptosis and cell death (Jung *et al*., 2007), the cytoprotective effects observed could be attributed to the presence of free radical scavenging compounds in the aqueous extract of *W. somnifera*. Evidence to support this mode of action has been shown previously. Inhibition of lipid peroxidation due to an antioxidant action has been observed both *in vitro* and *in vivo* with extracts of *W. somnifera* root and some of the component withanolides, including withaferin A (Dhuley, 1998; Panda *et al*., 1997; Bhattacharya *et al*., 2000; Chaurasia *et al*., 2000), a compound detected in the *W. somnifera* extract investigated in the present study. In addition to the withanolides having antioxidant capacity and decreasing lipid peroxidation in rodent brains, withanolides also enhanced catalase and glutathione peroxidase activities in rat frontal cortex and striatum (Chaurasia *et al*., 2000; Mishra *et al*., 2000; Scartezzini and Speroni, 2000; Bhattacharya *et al*., 1997 and 2001; Subbaraju *et al*., 2006). In another

study, an extract of *W. somnifera* was associated with attenuation of memory loss induced by oxidative stress mediated by free radicals in an animal model (Parihar *et al*., 2004)**.** *W. somnifera* root extract was also shown to significantly reduce the level of lipid peroxidation and improved the antioxidant status in an animal model of Parkinson's disease (Sankar *et al*., 2007). Other studies showed that *W. somnifera* root extract also imparted neuroprotective effects, observed in a 6-hydroxydopamine-induced model in rats, and a lithium-pilocarpine induced seizures model of status epilepticus in rats (Kulkarni *et al.*, 1998) and various other animal models of neurological disorders, including Parkinsonism and epilepsy (Sanker *et al*., 2007**;** Kulkarni and Dhir, 2008)**.** There is also evidence of neuroprotection by withanolide A, withanoside IV, and withanoside VI, steroidal derivatives isolated from a root extract of *W. somnifera*, that attenuated Aβ (25–35)-induced axonal, dendritic and synaptic losses and memory deficits in mice (Tohda *et al*., 2005; Kuboyama *et al*., 2005; Kuboyama et al., 2006). A study by Jayaprakasam *et al*. (2009) demonstrated the ability of withanamides A and C present in *W. somnifera* fruit extracts to protect undifferentiated PC-12 cells from Aβ cytotoxicity. Thus, the neuroprotective effects of the *W. somnifera* aqueous root extract against H_2O_2- and Aβ-induced cytotoxicity shown in this study, are consistent with other studies that have suggested *W. somnifera* may be neuroprotective *in vivo* by mediating antioxidant effects.

One *in vitro* study showed an interesting finding in which *W. somnifera* root extract significantly and dose-dependently increased the percentage of cells with neurites in human neuroblastoma SK-N-SH cells (Tohda *et al*., 2000). However, one observation in our study was that at high concentrations (> 100 µg/mL), the aqueous extract exhibited cytotoxic and perhaps anti-proliferative properties against the dPC12 cell line,

in line with reputed anticancer properties of *W. somnifera* (Subbaraju *et al*., 2006; Stan *et al*., 2008). Indeed, component **32**, detected in the *W. somnifera* extract by LC-MS^n analysis (Table 1, Fig. 5), showed mass spectral data consistent with the dimeric thiowithanolide, ashwagandhanolide, a compound reported to inhibit cell proliferation in various tumor cell lines (Subbaraju *et al*., 2006).

In conclusion, this study demonstrated that an aqueous extract of *W. somnifera* root, in which numerous withanolide derivatives were detected, was neuroprotective against H_2O_2- and Aβ-induced cytotoxicity, providing further evidence to explain the use of *W. somnifera* root in some cognitive disorders. However, further investigations are required to confirm the identity and potency of the specific compounds responsible for the reported neuroprotective effects of *W. somnifera* against H_2O_2- and Aβ-induced cytotoxicity, and to determine whether the observed neuroprotective effects are due to potential polyvalent activities (Williamson, 2001) amongst the constituents of the withanolide-rich aqueous extract.

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Figures legends

Fig. 1 Concentration-dependent inhibition of cell viability by H_2O_2 in differentiated PC12 cells after treatment for 24, 48 and 72 hr. Values are expressed as a percentage of control values. C: untreated control. Data are presented as the mean ± SEM of three separate experiments performed in triplicate. * p<0.05, **p<0.01 and ***p<0.001 compared with the untreated control. ■ 24 h ■ 48 h □ 72 h

Fig. 2 Protective effect of an aqueous extract of *W. somnifera* against H_2O_2-induced cytotoxicity in differentiated PC12 cells. NC: negative control (H_2O_2 alone, 200µM); PC: positive control (H_2O_2 absent). Values are expressed as a percentage of control (PC) values. Data are presented as mean ± SEM of three separate experiments

performed in triplicates. *p<0.05, **p<0.01 and ***p<0.001, compared with negative control. The protective effect of *W. somnifera* against H_2O_2-induced cytotoxicity was evaluated after 24 hr incubation time.

Fig. 3 Dose-dependent neurotoxicity of $A\beta_{(1-42)}$ aggregates in differentiated PC12 cells measured by an MTT assay (incubation time: 24 hr). Values are expressed as a percentage of control values. C: untreated control. Data are presented as the mean ± SEM of three separate experiments performed in triplicate. *p<0.05, **p<0.01 and ***p<0.001, compared with control.

Fig. 4 Protective effect of *W. somnifera* extract against $A\beta_{(1-42)}$-induced toxicity in differentiated PC12 cells. NC: negative control (Aβ only, 0.5µg/mL); PC: positive control (Aβ absent). Values are expressed as a percentage of control (PC) values. Data are presented as the mean ± SEM of three separate experiments performed in triplicate. *p<0.05, **p<0.01 and ***p<0.001, compared with negative control. The protective effect of *W. somnifera* against $A\beta_{(1-42)}$ induced cytotoxicity was evaluated after 24 hr incubation time.

Fig. 5 MS1 base ion chromatogram from LC-MS^n (positive ESI) analysis of *W. somnifera* root aqueous extract. Peaks are assigned to withanolides, as presented in Table 1; compound **36** is assigned as withaferin A by comparison with a reference standard of withaferin A.

Table 1. Compounds observed by LC-MS analysis of *W. somnifera* root aqueous extract. Data presented are the retention time (min), the major ion species found in positive and negative modes (and the assignment of these ions), the deduced molecular mass (M_r) and the suggested molecular mass of the aglycone (A) determined from the positive and / or negative ion MS2 spectra.

Compound	Retention time (min)	Positive ion (*m/z*)	Assignment	Negative ion (*m/z*)	Assignment	M_r	A	Assignment	Product ions following MS2 of positive ion species listed (*m/z*; %)
1	7.4	224	[M + H]+	-	-	223	NA	-	124 (100) 93 (17)
2	7.8	226	[M + H]+	-	-	225	NA	-	124 (100) 93 (20)
3	8.9	226	[M + H]+	-	-	225	NA	-	126 (100) 84 (43)
4	9.1	794	[M + H]+	792	[M - H]-	793	-	-	776 (10) 655 (100) 648 (55)
5	9.9	574	[M + H]+	572	[M - H]-	573	-	-	556 (100) 538 (85) 472 (90)
6	10.1	649	[M + H]+	647	[M - H]-	648	470	withanolide derivative	632 (100) 556 (40) 471 (57) 299 (17) 281 (7)
7	10.2	1112	-	-	-	-	-	-	unstable ion
8	10.6	832	[M + NH_4]+	859	[M + HCOO]-	814	472	withanolide glycoside	815 (5) 635 (7) 473 (100)
9	11.0	794	[M + H]+	792	[M - H]-	793	520	withanolide derivative	665 (100) 521 (80)
10	11.1	962	[M + NH_4]+	943	[M - H]-	944	458	withanolide trihexoside	945 (916) 983 (30) 621 (100) 459 (95)
11	11.3	592	[M + H]+	590	[M - H]-	591	470	withanolide derivative	575 (80) 574 (100) 471 (35) 435 (20) 299 (30) 281 (20)
12	11.5	778	[M + H]+	776	[M - H]-	777	470	withanolide derivative	760 (10) 649 (100) 632 (55) 556 (20) 471 (22) 299 (9) 281 (4)
13	11.6	940	[M + H]+	938	[M - H]-	939	470	withanolide derivative	922 (70) 886 (68) 865 (86) 649 (100) 471 (16)
14	11.8	721	[M + H]+	719	[M - H]-	720	470	withanolide derivate	703 (16) 592 (100) 574 (41) 471 (17) 299 (12) 281 910)
15	11.9	883	[M + H]+	881	[M - H]-	882	-	withanolide derivative	865 (91) 847 (71) 829 (100) 799 (47) 592 (20)
16	12.1	778	[M + H]+	776	[M - H]-	777	452	withanolide derivative	760 (7) 649 (100) 632 (64) 556 (18) 453 (15)
17	12.2	940	[M + H]+	938	[M - H]-	939	-	withanolide derivative	922 (55) 886 (63) 856 (62) 649 (100)
18	12.4	721	[M + H]+	719	[M - H]-	720	452	withanolide derivative	703 (44) 592 (65) 574 (100) 556 (30) 453 (8)
19	12.6	778	[M + H]+	776	[M - H]-	777	452	withanolide derivative	760 (8) 703 (28) 649 (100) 631 (81) 453 (11)
20	12.9	897	[M + H]+	895	[M - H]-	896	502?	withanolide derivative	879 (100) 689 (38) 503 (8) 486 (11) 468 (7)
21	13.0	800	[M + NH_4]+	827	[M + HCOO]-	782	458	withanolide dihexoside	783 (8) 621 (97) 459 (100) 441 (19) 423 (10)
22	14.0	800	[M + NH_4]+	827	[M + HCOO]-	782	458	withanolide dihexoside	783 (71) 621 (25) 459 (100) 441 (76) 423 (41) 405 (25)

23	14.1	934	[M + H]+	932	[M - H]-	933	-	-	916 (20) 726 (53) 579 (40) 562 (100) 544 (30) 518 (22) 356 (11)
24	14.2	715	[M + H]+	-	-	714	470	withanolide derivative	679 (33) 622 (53) 471 (100) 299 (33) 281 (15)
25	14.4	816	[M + NH_4]+	843	[M + HCOO]-	798	456	withanolide dihexoside	799 (6) 619 (30) 457 (100) 439 (64) 421 (35) 403 (6)
26	14.5	489	[M + H]+	-	-	488	-	withanolide	317 (100) 299 (37) 281 (16)
27	14.7	800	[M + NH_4]+	827	[M + HCOO]-	782	458	withanolide dihexoside	783 (6) 603 (44) 459 (8) 441 (100) 423 (8) 405 (9)
28	15.0	762	[M + H]+	760	[M - H]-	761	454	withanolide derivative	744 (7) 633 (100) 616 (61) 455 (24)
29	15.1	650	[M + NH_4]+	-	-	632	470	withanolide monohexoside	471 (100)
30	15.2	638	[M + NH_4]+	-	-	620	458	withanolide monohexoside	621 (7) 603 (14) 459 (100) 441 (72) 423 (27) 405 (12)
31	15.4	762	[M + H]+	760	[M - H]-	761	454	withanolide derivative	744 (7) 633 (76) 615 (100) 455 (34)
***32**	15.5	976	[M + H]+	974	[M - H]-	975	-	-	772 (23) 604 (100) 417 (25)
33	15.8	654	[M + NH_4]+	681	[M + HCOO]-	636	458	withanolide monohexuronide	457 (100) 439 (53) 421 (19) 403 (3)
34	16.3	705	[M + H]+	703	[M - H]-	704	454	withanolide derivative	687 (20) 455 (26) 437 (94) 419 (100) 401 (67)
35	17.0	784	[M + NH_4]+	811	[M + HCOO]-	766	442	withanolide dihexoside (indicative of withanoside V)	767 (20) 605 (19) 443 (100) (425 (24) 407 (7)
****36**	17.4	471	[M + H]+	-	-	470	-	withaferin A	435 (10) 299 (74) 281 (100) 175 (6)
37	18.3	730	[M + H]+	728	[M - H]-	729	-	-	712 (20) 601 (41) 583 (100) 423 (77) 405 (48)
38	18.6	471	[M + H]+	-	-	470	-	withanolide	453 (15) 435 (100) 417 (94) 399 (63) 371 (17) 263 (49) 211 (22) 197 (13)
39	19.4	569	[M + H]+	567	[M - H]-	568	488	withanolide sulphate	533 (17) 515 (28) 497 (32) 453 (73) 435 (91) 417 (78) 397 (49) 361 (100) 283 (20)
40	19.8	488	[M + NH_4]+	-	-	470	-	withanolide	471 (65) 453 (100) 435 (32) 417 (71) 399 (54) 289 (23) 263 (36)
41	20.3	569	[M + H]+	567	[M - H]-	568	488	withanolide sulphate	489 (5) 453 (5) 397 (100) 317 (11) 299 (6) 281 (6)
42	22.8	586	[M + NH_4]+	567	[M - H]-	568	488	withanolide sulphate	569 (44) 551 (100) 533 (15) 515 (15) 453 (17) 417 (40) 399 (15) 389 (18) 361 (11)
43	23.4	280	[M + H]+	-	-	279	-	-	263 (100)
44	24.1	569	[M + H]+	567	[M - H]-	568	488	withanolide sulphate	ionisation in positive mode too low

*The dimeric thiowithanolide, ashwagandhanolide, with the same M_r as **32** has been reported by Subbaraju *et al*. (2006).
**Peak assigned as withaferin A by comparison with a reference standard for withaferin A

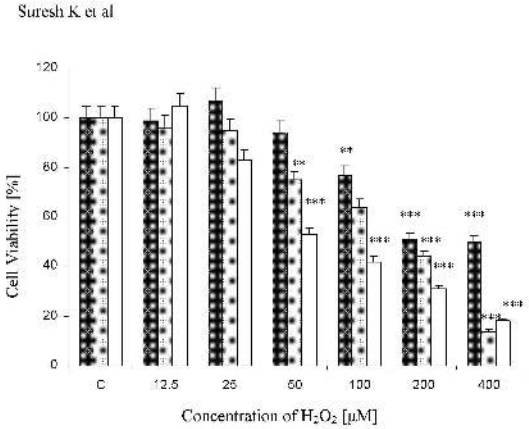

Fig. 1

209x297mm (300 x 300 DPI)

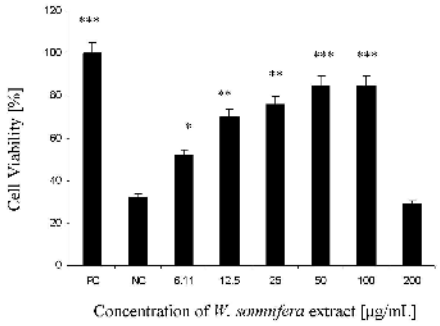

Fig.2

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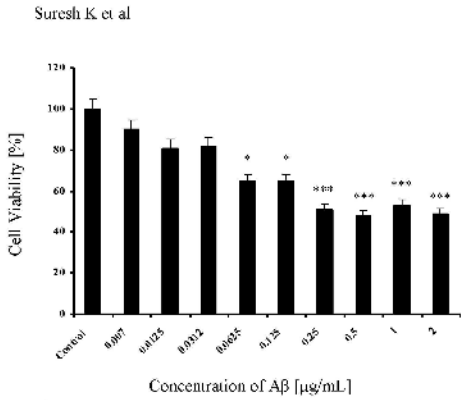

Fig. 3.

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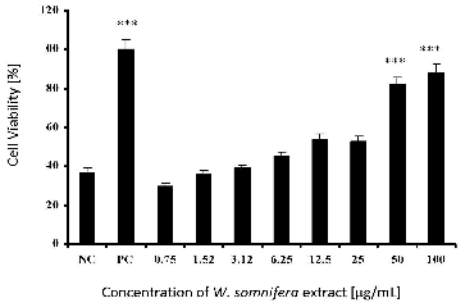

Fig. 4

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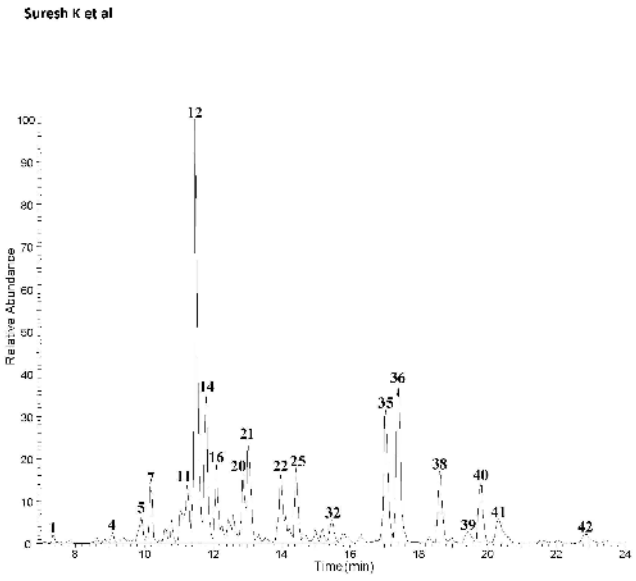

Fig. 5.

209x297mm (300 x 300 DPI)