Neuroprotective effect of *Withania somnifera*

**INTRODUCTION**

Acute ischemic stroke is the leading cause of death worldwide and is one of the main causes of long-term disability (Banerjee *et al.*, 2006). A widely applicable treatment for cerebral ischemia would have an enormous impact on public health. So far, the only beneficial treatment that has been developed is intravenous tissue type plasminogen activator (IV tPA) (Sila, 1993). Therefore, finding therapeutic compounds for the effective treatment of stroke is important.

Herbal remedies have recently emerged into prominence due to the lack of efficacy of conventional medicines. The Indian traditional health care system, Ayurveda, considers more than 2,000 plant species to have medicinal value (Gupta *et al.*, 2007; Mishra *et al.*, 2000).

It is important to note that *W. somnifera* can be used for treatment of disorders in all age groups, in both sexes and even during pregnancy, without any side effects (Sharma *et al.*, 1985). Previous studies have established the chemical composition and pharmacological and therapeutic efficacy of the plant (Bhatta *et al.*, 1987).

Withanolide derivatives isolated from the methanolic extract of *W. somnifera* have been shown to induce neurite outgrowth in the SH-SY5Y cell line (Zhao *et al.*, 2002) and induce expression of nitric oxide synthase (Iuvone *et al.*, 2003). Withanolide A is known...
to act as memory enhancer (Kuboyama et al., 2005). Despite its proven efficacy, there are very few studies regarding the evaluation neuroprotective role of *W. somnifera* against ischemic stroke (Adams et al., 2002; Chaudhary et al., 2003).

Pheochromocytoma (PC12) cells have been widely used for invitro studies of neuronal cells (Abu et al., 1993; Henriques et al., 2005). Therefore in the present study, we have evaluated neuroprotective effect of *W. somnifera* against in vitro chemical ischemia/reperfusion in PC12 cells using XTT assay, LDH release assay and DNA fragmentation assay. The antioxidant properties of *W. somnifera* extract was also investigated by DPPH assay.

**MATERIALS AND METHODS**

The PC12 cell line was procured from the National Center for Cell Science (NCCS) (Pune, Maharashtra, India). Dulbecco’s modified eagle medium (DMEM), sodium azide, phosphate buffered saline and antibiotic solutions were obtained from Himedia, India. Heat inactivated horse serum, newborn calf serum, 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolim-6-carboxanilide inner salt (XTT), 1,1-diphenyl-2-picyryldiazyl radical (DPPH), cetyl-trimethyl-ammonium bromide (CTAB) and other chemicals were purchased from Sigma Co. (St. Louis, MO, USA). Cell culture plastic wares were purchased from Axiva Sichem Pvt. Ltd. (New Delhi, India).

**Culture of PC12 cells**

PC12 cells were routinely maintained in DMEM medium supplemented with 10% heat inactivated horse serum, 5% newborn CalFs. Serum, 10μg/ml penicillin, 25 μg/ml streptomycin and 25μg/ml amphotericin B (complete DMEM medium). Cells were routinely sub-cultured every 4-5 days.

**Herbal extract**

Commercial total root extract of *W. somnifera* was purchased from the manufacturer Innocon Pharmaceutical Pvt. Ltd. (Pune). The supplied extract was prepared by multiple solvent extractions using a methanol: isopropyl alcohol: acetone preparation. *W. somnifera* extract was also used as a stock solution (concentration of extract was approximately 10 mg/ml).

**Induction of chemical ischemia/reperfusion and application of *W. somnifera* extract**

Tissue culture plates (24-well) were coated with Poly-L-Lysine (Sigma, St. Louis, MO). PC12 cells were seeded into Poly-L-Lysine coated 24-well tissue culture plates at a density of 5 x 10^5 cells/well and incubated for 48 hrs. After 48 hrs, cells were washed twice with glucose-free CaKR Buffer. PC12 cells were exposed to ischemic medium (CaKR buffer containing 10mM sodium azide) for three hours in the presence of *W. somnifera* extract at concentrations of 100, 200, 400, and 1000μg/ml. As a control, PC12 cells were also exposed to ischemic medium without *W. somnifera* extract added. After three hours, the exposure media was removed and cells were washed with CaKR buffer twice. Normal cell culture media (complete DMEM medium) are then added and incubated for 24 hrs to allow for reperfusion.

**Cell viability assay**

Cell viability was assessed using the XTT assay as described earlier (Deshpande et al., 2007). Briefly, after 24 hours of incubation for reperfusion, 20 μl of XTT solution (1 mg/ml) containing phenazine methosulfate (PNS) (0.92 mg/ml XTT solution) was added to each well. After a four-hour incubation period, the plate was read at a wavelength of 450 nm on a Stat Fax 325+ microtiter plate reader (Ark Diagnostic, Mumbai, India). Mean background values were obtained by scanning wells containing medium only.

**Trypan blue dye exclusion assay**

At the end of a 24-hr reperfusion period, the culture medium was removed and replaced with a 0.1% Trypan blue solution in phosphate buffered saline for 5 minutes at ambient temperature. The dye solution was then removed and photographs were taken on an inverted tissue culture microscope (mvtex, India) to assess for protection by *W. somnifera* extract.

**LDH release assay**

Quantification of membrane permeability and extent of cell damage was done using the LDH release kinetic assay as previously described (Wróblewski et al., 1955). Briefly, culture supernatants were collected after reperfusion. The reactions was initiated by mixing 100 μl of supernatant or pellet with 2.6 ml of substrate solution [potassium phosphate buffer 2.4 ml + 100μl of NADH2 (5 mg/2 ml) + 100μl sodium pyruvate (5 mg/2 ml)]. LDH activity was measured at 340 nm on a UV-Vis spectrophotometer (108 Systronics, India). Activity is expressed as Units/5x10^5 cells.
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DNA fragmentation assay
For gel analysis of DNA fragmentation, PC12 cells were harvested after reperfusion, 30 μl 10% SDS and 3 μl proteinase K (20 mg/ml) was added and the solution was mixed. This mixture was incubated at 37°C for 2 hours. After incubation, 100 μl of 5 M NaCl and 80 μl of high-salt CTAB buffer (containing 4 M NaCl and 1.8% CTAB) were added and mixed. This solution was incubated at 65°C for 10 min. An approximately equal volume of (0.7-0.8 ml) of chloroform-isoamyl alcohol (24:1 v/v) was then added, mixed thoroughly and centrifuged for 4-5 min in a microcentrifuge at 12,000 rpm. The aqueous viscous supernatant was carefully decanted and transferred to a new tube and equal volume of phenol: chloroform-isoamyl alcohol (1:1 v/v) was added. This solution was centrifuged for 5 minutes at 12,000 rpm. Supernatant was separated and mixed with 0.6 volume of ice-cold isopropanol and kept at -20°C overnight to obtain a precipitate. Precipitated DNA was centrifuged, air-dried, and resuspended in 20 μl of distilled water. DNA fragments were fractionated on a 1.5% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide and photographed under UV light.

DPPH assay
Antioxidant activity of W. somnifera was tested using the DPPH radical assay, as previously described (Goupy et al., 1999). Briefly, 0.6 ml of W. somnifera extract solution (1000 μg/ml) or water (reference sample) was added to 0.6 ml of methanolic DPPH solution (100 μM). Absorbance of the DPPH radical was recorded at 517 nm using a spectrophotometer after 30 minute incubation. Radical scavenging activity of the extract was expressed as percentage of inhibition (PI) of radical absorbance. PI was calculated using the absorbance of the reference reaction to represent a 100% absorbance of DPPH radical.

Statistical analysis
Statistical evaluations of neuroprotective effects were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s tests, while statistical differences in antioxidant activity was calculated by paired sample t-test. All statistical analysis was performed using MedCalc statistical software. P Value <0.05 were considered as statistically significant. Linear regression analysis was used for calculation of ED50.

RESULTS
Effect of Withania somnifera extract on PC12 cell viability
Viability of PC12 cells was assayed using an XTT assay. First, we evaluated the toxicity of Withania somnifera extract at concentrations of 100-1000 μg/ml and found that the extract was not toxic to PC12 cells in this range of concentrations (Fig. 1 [Supplementary data]). Viability of PC12 cells is significantly decreased after ischemia/reperfusion (Isc-R) (p<0.0008 vs. control). Addition of Withania somnifera extract protects against PC12 cell death in a dose-dependent manner, and showed significant protection at concentrations of 400 and 1000 μg/ml (p<0.0013 and 0.0004 respectively vs. Isc-R) as shown in Fig. 2 [Supplementary data]. Effective dose of Withania somnifera extract which gives 50% protection (ED50) against chemical ischemia/reperfusion in PC12 cell was calculated by linear regression of percentage protection vs. extract concentration (y =32.894 Ln (x) – 137.19, R2=0.9353), the calculated ED50 was found to be 295.89 μg/ml (Fig. 4 [Supplementary data]).
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The effect of different concentrations of *W. somnifera* on cell membrane damage was studied using the LDH release assay. A significant increase in LDH activity was observed after ischemia/reperfusion (p<0.0001 vs control). *W. somnifera* was found to prevent ischemia/reperfusion induced membrane damage in PC12 cells in dose-dependent manner, with significant effects observed at concentrations of 200, 400, and 1000 µg/ml (Fig. 5 [Supplementary data], p<0.0002, 0.0001, <0.0001 respectively vs. Isc-R).

Effect of *W. somnifera* extract on PC12 cell apoptosis

In this study, we observed intact chromosomal DNA in control PC12 cells (Fig. 6A, lane 1 [Supplementary data]) while DNA damage was seen PC12 cells after chemical ischemia/reperfusion (Fig. 6A, lane 2). Anti-apoptotic activity was monitored after addition of *W. somnifera* extract in tested concentrations and it was found that the extract protects PC12 cells against apoptotic cell death at concentrations of 400 and 1000 µg/ml (Fig. 6A, lanes 5 and 6).

Free radical scavenging activity of *W. somnifera*

DPPH (an inducer of stable free radicals) was used to assess radical-scavenging activity of *W. somnifera* extract. Administration of *W. somnifera* extract (1000 µg/ml) reduced DPPH-induced free radical activity by 65% (p<0.0025), as shown in Fig. 6B [Supplementary data].

DISCUSSION

In ischemic stroke damage occurs through multiple mechanisms (Lipton, 1999). Therefore therapeutic agent who acts via multiple mechanisms would be the better neuroprotective agent. Studies with *W. somnifera* have indicated that it exerts an anti-ageing effect and has anxiolytic and anti-depressant activity (Bhattacharya *et al.*, 2000). The other pharmacological actions exerted by *W. somnifera* include anti-inflammatory, anti-stress, hemopoietic immunomodulatory, and anti-oxidant effects (Bhattacharya *et al.*, 2001)

Objective of the present study was to investigate the neuroprotective role of *W. somnifera* extract against *in vitro* chemical ischemia/reperfusion in PC12 cells. We found that *W. somnifera* extract was not toxic to PC12 cells. In fact, application of *W. somnifera* extract was observed to increase PC12 cell survival (as shown by XTT assay and trypan blue dye exclusion assay) and
reduce LDH release after ischemia/reperfusion. These results suggest that *W. somnifera* extract offers protective effects against *in vitro* chemical ischemia/reperfusion in PC12 cells. Earlier Chaudhary *et al.* (2003) have demonstrated that *W. somnifera* protects rat from middle cerebral artery (MCA) occlusion. Thus our result confirms earlier report and this is a first report on neuroprotective effect of *W. somnifera* in *in vitro* cell line model of ischemia. Previous studies have confirmed that apoptosis is one of the major routes of pathological neuronal death during ischemic injury (Chen *et al.*, 1998; Tabakman *et al.*, 2004). DNA fragmentation is frequently used as a marker of apoptosis (Kuboyama *et al.*, 2005). *W. somnifera* extract was shown to have an anti-apoptotic effect (Hamza *et al.*, 2008). We found that *W. somnifera* prevents DNA fragmentation induced by chemical ischemic insult in PC12 cells thus confirms the anti-apoptotic effect of *W. somnifera*. This suggest that *W. somnifera* extract may offer protection against ischemic/ reperfusion to PC12 cells via an anti-apoptotic mechanism.

Excessive production of reactive oxygen species (ROS) is also believed to play a critical role in development of ischemic brain injury (Chan *et al.*, 1996; Ozben *et al.*, 1998). *W. somnifera* extract was reported to have a significant anti-oxidant activity (Kaur *et al.*, 2004; Bhatnagar *et al.*, 2005). We have also found that root extract from *W. somnifera* has significant antioxidant properties using the DPPH assay.

From above observation it can be concluded that *W. somnifera* extract protect PC12 cells against chemical ischemia/reperfusion damage as shown by the XTT assay and LDH release assay. *W. somnifera* extract protects PC12 cells via an anti-apoptotic mechanism, as shown by DNA fragmentation assay. *W. somnifera* also has significant antioxidant activity, which may contribute to the protection of PC12 cells against chemical Ischemia/reperfusion. Thus *W. somnifera* extract may be useful in the therapeutic management of ischemic stroke. Further studies are required, using active constituents isolated from *W. somnifera*, in order to determine if one or more bioactive compounds present in this herb act synergistically to offer protection against chemical ischemia/reperfusion.

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**References**


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