Original Article

In vitro Neuroprotective Effect of *Valeriana wallichii* Extract Against Neurotoxin and Endoplasmic Reticulum Stress Induced Cell Death in SH-SY5Y Cells

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ABSTRACT

Objectives: To investigate the neuroprotective efficacy of *Valeriana wallichii* DC rhizome extract against 1-methyl-4-phenlypyridinium (MPP^+) and tunicamycin induced cell death. **Methods:** The 50% methanolic extract of *V. wallichii* rhizome (VWE) was evaluated for free radical scavenging properties. Cell viability analysis and IC_{50} determination for VWE in SH-SY5Y neuroblastoma cell line was performed by MTT assay. The neuroprotective efficacy of VWE in SH-SY5Y cells against the damages induced by the neurotoxin, MPP^+ and the endoplasmic reticulum stress inducer, tunicamycin was assessed by MTT assay and by morphological analysis with phase contrast microscope. MTT assay was performed using 3 different concentrations (0.1, 0.5 and 1 mg/mL) of VWE that were added at 0, 8 and 16 h post MPP^+ and tunicamycin treatment. **Results:** VWE showed good free radical scavenging properties. From the cell viability analysis the IC_{50} value of VWE was calculated as 2.207 mg/mL in SH-SY5Y cells. In MPP^+ treated cells 0.5mg and 1mg/mL concentrations of VWE brought significant improvement in cell viability at 0h, while at 8h and 16h post MPP^+ treatment the effect was significant at 1mg/ml concentration. VWE exhibited significant protective effect in tunicamycin treated cells only at 0h but not at 8h and 16h post treatment. MPP^+ and tunicamycin induced cell shrinkage and condensation were inhibited by treatment with 1mg/mL of VWE. **Conclusions:** The present work is the first study that sheds light on the cytoprotective effect of *Valeriana wallichii* DC rhizome and provides lead for identification and isolation of novel drugs for various neurodegenerative diseases.

Keywords: *Valeriana wallichii* DC; SH-SY5Y neuroblastoma; MPP^+; Tunicamycin; MTT.

INTRODUCTION

The genus *Valeriana* contains about 230 species. The 3 most important species that play a role in herbal medicine are *V. officinalis* L, *V. wallichii* DC (synonym *V. jatamansi* Jones) and *V. edulis* ssp. *procera* Meyer (synonym *V. mexicana* DC). In Europe *V. officinalis* is cultivated for phytomedicine preparations. *V. wallichii* DC, the Indian valerian, is native to the Himalayas. *V. edulis* ssp. *procera* Meyer originates from Central America. The Greek and the Roman physicians have used valerian root as a diuretic, pain relieving and spasmolytic agent. Nowadays, valerian preparations are used primarily as a mild sedative, to treat neurasthenia and emotional stress[1]. Valerian is a highly respected medicinal plant described in many pharmacopoeia monographs. Radix Valerianae (Subterranean parts of *V. officinalis* L) is included in the WHO monograph[2].

Indian Valerian, tagar, (*V. wallichii* DC) has been used in Indian herbal medicines, and is considered as a substitute of the European *V. officinalis*[3]. The roots are highly aromatic. The active principles are Valpotriates, dihydrovaltrate, isovalerianate, 6-methylapigenin, hesperidin and sesquiterpenoids. Its rhizome and root are known to have alkaloids, bornyl isovalerianate, chatinine, formate, glucoside, isovalerenic acid, 1-camphene, 1-pinene, resins, terpineol, valerianine, citric acid, malic acid, maliol, succinic acid and tartaric acid[4].

In Chinese folk medicine *V. wallichii* is used for skin infections and for its activities on central nervous system[5,6]. It is one of the herbs mentioned in all scriptures of Ayurveda. In Dhanvantari Nighantu and Bhavaprakasha its activity is cited on the central nervous system[7]. Charak Samhita has described it as cure for snake poisoning. It is used in Unani and Ayurvedic medicines for treating obesity, insanity and epilepsy. The entire plant is used for nervous debility, failing reflexes, as hypnotic and in the treatment of spastic disorders[8]. Dhanya Panchaka Kashaya (An herbal preparation) containing *V. wallichii* has been found to be effective in dyspeptic symptoms[9]. Many of the phytoconstituents of *V. wallichii*, like 6-methyl apigenin, hesperidin, iridoids have stimulatory or protective effects on CNS[10,11].

This work reports the *in vitro* antioxidant effects of 50% methanolic extract of *V. wallichii* rhizome (VWE) and its neuroprotective effect against the neurotoxin, 1-methyl-4-phenlypyridinium (MPP^+) and tunicamycin (Endoplasmic reticulum stress inducer) induced cell death in SH-SY5Y neuroblastoma cells.

MATERIALS AND METHODS

Chemicals and reagents

MPP^+ and Tunicamycin were purchased from Sigma-aldrich, Bangalore, India. All other chemicals and reagents used in this work were of analytical or cell culture grade and purchased from HiMedia, India.

Preparation of plant extract

The *Valeriana wallichii* rhizome was purchased from local market, Delhi, India. The samples were authenticated by Taxonomist, Department of Botany, Loyola College, Chennai, India and a voucher specimen has been maintained in our lab. For preparation of extract, the dried rhizome was ground and mixed with 50% methanol in the ratio of 1:5 (w/v) and kept at 150 rpm in an orbital shaker for 24h and filtered through Whatmann No.1 filter paper. The filtrate was condensed in rota evaporator and lyophilised. This lyophilised sample was used for the subsequent studies.

In vitro free radical scavenging assays

1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging activity

The DPPH· radical scavenging activity of the rhizome extract in varying concentrations (10, 50, 100, 150, 200, 250 and 500µg/ml) was evaluated as described previously[12]. The results are expressed as % DPPH radical scavenged, calculated by the formula:

% of DPPH radical scavenged = [(A Control – A Sample) / A Control] × 100

Where A is absorbance at 517nm[12]

Nitric Oxide Scavenging activity

The nitric oxide scavenging activity was assayed by the method of Marcocci *et al*[13]. Briefly, 0.3 ml of sodium nitroprusside was added to tubes containing 0.1 ml of various concentrations of 50% methanolic extract of *Valeriana wallichii* rhizome (VWE) (10, 50, 100, 150, 200, 250 and 500µg/ml) and incubated for 150 min. After incubation, 0.4 ml of Griess reagent was added to each sample and the absorbance was measured at 546 nm against control samples. Results were recorded as percentage inhibition of nitrite formed.

Superoxide anion radical scavenging activity

Superoxide radical was generated *in vitro* by a non-enzymatic method involving the nicotinamide adenine dinucleotide- nitro blue tetrazolium - phenazine methosulphate (NADH- NBT - PMS) system following the procedure of Nishikimi *et al*[14]. Varying concentrations (10, 50, 100, 150, 200, 250 and 500µg/ml) of VWE as added to tubes containing 50 µM NADH followed by addition of 150 µM of NBT. The reactions were initiated by adding PMS (15 µM) and the absorbance was read at 560 nm exactly 1 min later. Results were recorded as percentage inhibition of superoxide.

Hydrogen peroxide (H2O2) Scavenging activity

The ability of VWE to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*[15]. VWE of different concentrations (10, 50, 100, 150, 200, 250 and 500µg/ml) was added to fresh H_2O_2 solution (0.6 mL, 40mM). Absorbance of H_2O_2 at 230 nm was measured after 10 minutes against a blank solution without H_2O_2. Results were represented as percentage of H_2O_2 scavenged.

Cell culture

Human neuroblastoma SH-SY5Y cells were obtained from NCCS, Pune, India. The cells were cultured in DMEM: HAM F-12 medium supplemented with 10% fetal bovine serum, 100 UI/ml penicillin and 100 µg/ml streptomycin at 37ºC in a humidified atmosphere of 5% CO_2. The culture medium was changed every 2 days. Cells were seeded in 96-well plates at a density of $10x10^3$ cells / well and were cultured for 48 h.

Half maximal inhibitory concentration (IC50) determination for VWE in SH-SY5Y cells

To arrive at the IC_{50} value for VWE in SH-SY5Y cells, the cells were treated with various concentrations of VWE (0.05, 0.1, 0.5, 1.0 and 5mg/mL) and incubated for 24h. Cell viability was examined by MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetra-zolium bromide) assay. Briefly, following VWE treatment and 24hr incubation at 5% CO_2, MTT was added to each well and further incubated for 3h in dark. After incubation 100µl of DMSO was added to dissolve the purple coloured formazan crystals formed and the absorbance was measured at 570 nm[16] with a micro plate reader. Wells without cells were used as blanks and the results are expressed as % viable cells. Data obtained from this experiment were fitted to a non-

linear regression plot to determine the IC_{50} using Origin pro 8 software.

Effect of VWE against MPP+ and tunicamycin induced damage in SH-SY5Y cells

Cells were treated with MPP^+ as per the procedure described by Christopher P. Fall and James P. Bennett, Jr.[17]. To treat the cells with tunicamycin, the procedure described by Vibeke Hervik Bull and Bernd Thiede was followed[18]. Following the addition of 5mM MPP^+ /10µM tunicamycin cells were treated with varying concentrations (0.1, 0.5 and 1mg/mL) of VWE at 0, 8 and 16h post toxin addition. After 24h the Cell viability was analysed by the MTT method. Cell morphology was analysed using phase contrast microscope.

Statistical analysis

All the data were analyzed using the SPSS 7.5-Windows Students version software (SPSS Inc., Chicago, IL, USA). For all the measurements, one-way ANOVA followed by Tukey's test was used to assess the statistical significance between groups. A statistically significant difference was considered at the level of $*p \leq 0.05$ and $**p \leq 0.001$.

RESULTS AND DISCUSSION

Herbs play a significant role in improving and maintaining human health. Several medicinal plants have been proved to exhibit good *in vitro* free radical scavenging activities[19] and neuroprotective effects[20]. Plethora of bioactive phytochemicals with antioxidant[21], neuroprotective[22], anticancer[23], anti-inflammatory[24] and anti-HIV properties[25] have been identified from traditionally used medicinal plants as well as from commonly used spices. In the present study, 50% methanolic extract of *V. wallichii* rhizome was investigated for its potential radical scavenging and neuroprotective effects.

In vitro free radical scavenging activities

DPPH Radical Scavenging activity

DPPH is a free radical that has been widely used to study the scavenging activities of several compounds including plant extracts. The DPPH radical is scavenged by antioxidants through the donation of a hydrogen atom or electron forming the reduced DPPH. DPPH radical quenching effect of VWE showed an increasing trend with increasing concentrations of VWE (Fig. 1.).

Nitric Oxide Scavenging activity

In this test Nitric oxide ($NO^{\cdot}$) free radical generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions that are measured by Griess reagent. The ability of VWE to inhibit nitrite ion formation was found to increase with increasing concentrations of the extract (Fig. 2.). VWE competes with oxygen to react with Nitric oxide thereby inhibiting generation of nitrite ions.

Superoxide anion radical scavenging activity

In this assay superoxide anions are generated by PMS in aqueous solution which reduce NBT to form blue colored formazan crystals that are measured at 560nm. VWE interfered this reaction by interacting with superoxide anions and the percentage inhibition was found to increase with increasing concentrations of VWE as shown in Fig. 3.

H2O2 Scavenging activity

VWE was found to be effective in quenching the H_2O_2 free radicals also and the

effect was observed to increase with increasing concentrations of VWE (Fig.4.).

Thus, in the present study 50% methanolic extract of *Valeriana wallichii* rhizome (VWE) showed tremendous Superoxide, DPPH and NO scavenging activities and comparably moderate H_2O_2 scavenging activity. The radical scavenging properties were found to improve with increasing concentrations of VWE. Reports are available on the free radical scavenging potential of *V. wallichii* aqueous and methanolic extracts[26-28]. This may be attributed to the presence of phytocompounds like flavonoids and alkaloids.

IC50 determination in SH-SY5Y cells

SH-SY5Y neuroblastoma cells can be differentiated into neuron-like cells displaying morphological and biochemical features of mature neurons and hence have been widely used as model systems in neurodegenerative disease studies and in other neuroscience research[29]. In the present study SH-SY5Y cells were treated with varying concentrations of VWE ranging from 0.05 mg/mL to 5 mg/mL as shown in Fig. 5A to examine toxic effects if any. Although the % SH-SY5Y cell viability was reduced ($p \leq 0.05$) at 0.05 and 0.1mg/ml concentrations of VWE, the effect was more pronounced at higher doses of 0.5, 1 and 5mg/mL concentrations ($p \leq 0.001$). The IC_{50} for VWE was arrived from the dose response curve and was found to be 2.207 mg/mL (Fig. 5B) which suggests that VWE exhibit toxic effects only at higher concentrations. In an earlier report, similar observations were made with aqueous extract of *V. officinalis* which showed an IC_{50} value of 2.805mg/ml in SH-SY5Y cells[30].

Time and dose dependent protection of SH-SY5Y cells by VWE against MPP+ induced neurotoxicity

The neurotoxin MPP^+, an active metabolite of MPTP (N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) is known to induce Parkinsonism in humans and primate models. The uptake of MPP^+ ions by dopaminergic neurons is found to take place via dopamine transporter (DAT). Norepinepherine transporter expressed by SH-SY5Y cells is known to transport both dopamine and MPP^+ at a slower rate than DAT. When MPP^+ enters the cell it is concentrated into the mitochondria where it inhibits complex I of the mitochondrial electron transport chain. MPP^+ induced initiation of apoptosis in SH-SY5Y cells is found to involve mitochondrial damage mediated oxidative stress[17]. In this study, MPP^+ treatment resulted in extensive cell death and only 12.17% of SH-SY5Y cells were found to be viable after 24h of treatment (Fig. 6.). Remarkably, treatment with VWE showed impressive improvement in cell viability when added along with MPP^+ (0 h) and the percentage viability increased with increasing concentrations of VWE with 0.5 mg VWE showing 37% cell viability ($p \leq 0.05$) and 1mg/mL VWE showing 64% cell viability ($p \leq 0.001$). This indicates that VWE could bring about protective effects against MPP^+ induced damage when treated at the same time along with the toxin. On the other hand, VWE treatment was found to be less effective in reverting MPP^+ induced damage when added at 8 and 16 h post MPP^+ treatment with 1mg/mL concentration of VWE exhibiting cell viability of 44% and 36% ($p \leq 0.05$) respectively (Fig. 6). Similar *in vitro* cytoprotective effects were observed with *V. officinalis* against rotenone induced damage in SH-SY5Y cells[30]. The observed cytoprotective effect of VWE can be attributed to its free radical scavenging properties that is substantiated by the *in vitro* tests performed in this study. Our findings commemorate the previous report on the neuroprotective effect of *V. wallichi* extract in ischemia and reperfusion induced cerebral injury in mice[31]. Reports are also available on the protective effect of phytocompounds of *V.*

wallichii against MPP^+ induced SH-SY5Y cell death[6,11].

Time and dose dependent protection of SH-SY5Y against tunicamycin induced cell death by VWE

The eukaryote organelle endoplasmic reticulum (ER) is responsible for synthesis and maturation of membrane and secretory proteins. Conditions, that affect the protein folding process lead to the accumulation of unfolded or misfolded client proteins in the ER lumen, and triggers a set of signalling pathways called ER stress/unfolded protein response (UPR)[32]. Protein aggregation and ER stress has been associated with neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease resulting in neural cell death[33]. Tunicamycin is a bacterial toxin that inhibits N-linked glycosylation of nascent proteins resulting in ER stress and activation of UPR in mammalian cells. Increased expression of ER stress markers like Bip, CaBp, CHOP etc., is reported in tunicamycin treated SH-SY5Y cells[18]. In the present study, tunicamycin treatment of SH-SY5Y cells induced formidable cell death with only 18% of cells being viable after 24h (Fig. 7). Grippingly, VWE treatment at 0 h protected against tunicamycin induced cell death and the % cell viability was found to be 36, 45 and 91% at 0.1, 0.5 and 1mg/mL concentrations respectively. Whereas, VWE was found to be less effective when added after 8 and 16 h post tunicamycin treatment (Fig.7). Ours is the first report on the protective effect of VWE against tunicamycin induced cell death in SH-SY5Y cells and hence against ER stress mediated cell death.

Effect of VWE on cellular morphology of SH-SY5Y cells treated with MPP+ and tunicamycin

Significant changes in cellular morphology were observed when cells were treated with 5mM MPP^+ or 10 µM tunicamycin for 24 h. In MPP^+ treatment, surviving SH-SY5Y cells showed retraction of cytoplasm and decrease in tack on the plate, that was different from the typical morphology. This effect was partially reverted when the VWE extract (1mg/mL) was added at the same time of MPP^+ addition (0h) (Fig.8A.). In tunicamycin treatment, surviving cells showed severe retraction of neuronal outgrowth and the cells exhibited rounded morphology. This effect was reverted with the addition VWE extract (1mg/mL) at the same time of tunicamycin addition (0h). In these groups, the normal morphology of surviving SH-SY5Y cells being almost totally recouped (Fig.8B.).

CONCLUSION

The results from this study bring to the surface the potent neuroprotective property of *V. wallichii* rhizome against MPP^+ and tunicamycin induced cell death. The observed effect of this exotic plant can be further analysed in different models of neurodegenerative diseases and the plant can be used to develop new drugs that can alleviate such diseases.

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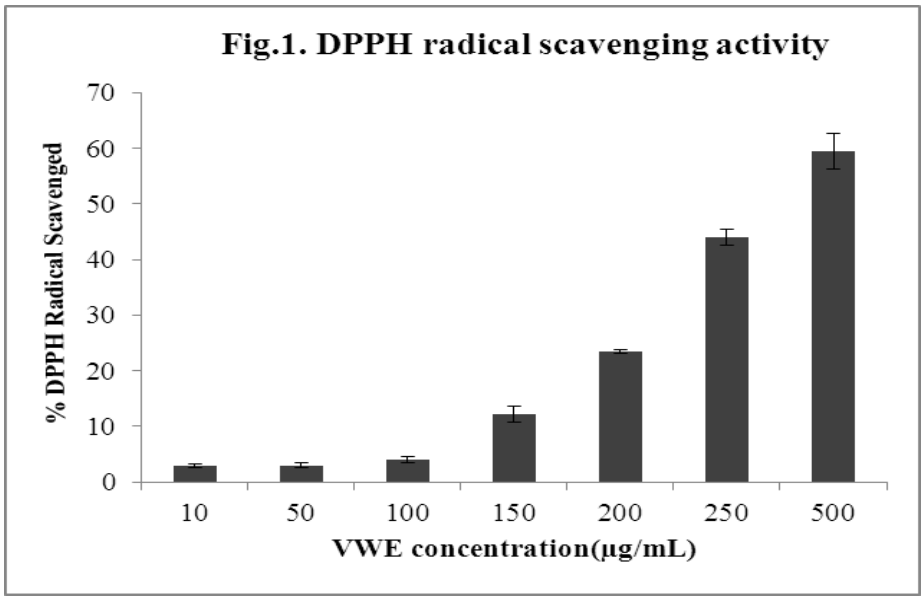

Figure 1. DPPH· Radical Scavenging activity of different concentrations of VWE

Values are expressed as mean ± SE of three independent experiments.

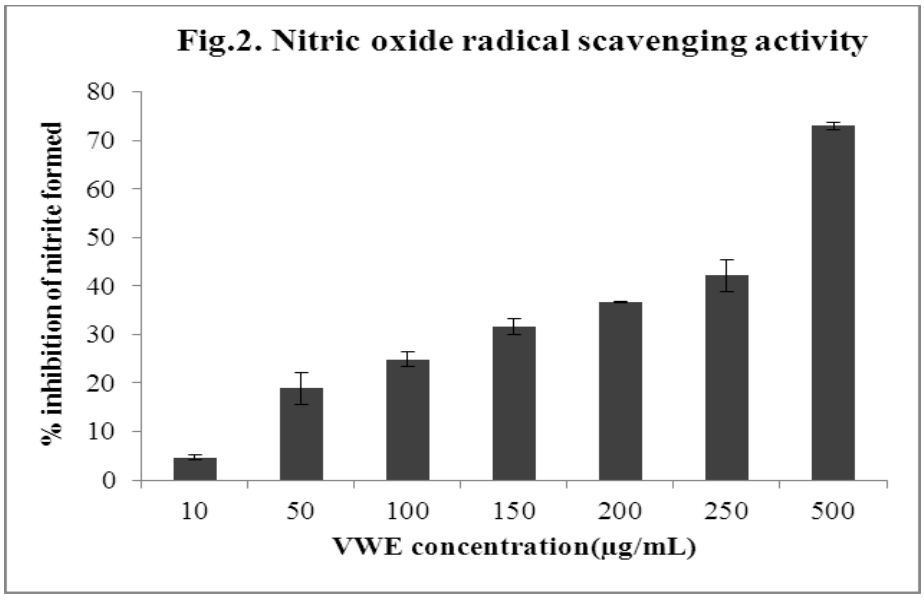

Figure 2. NO· Radical Scavenging activity of different concentrations of VWE

Values are expressed as mean ± SE of three independent experiments.

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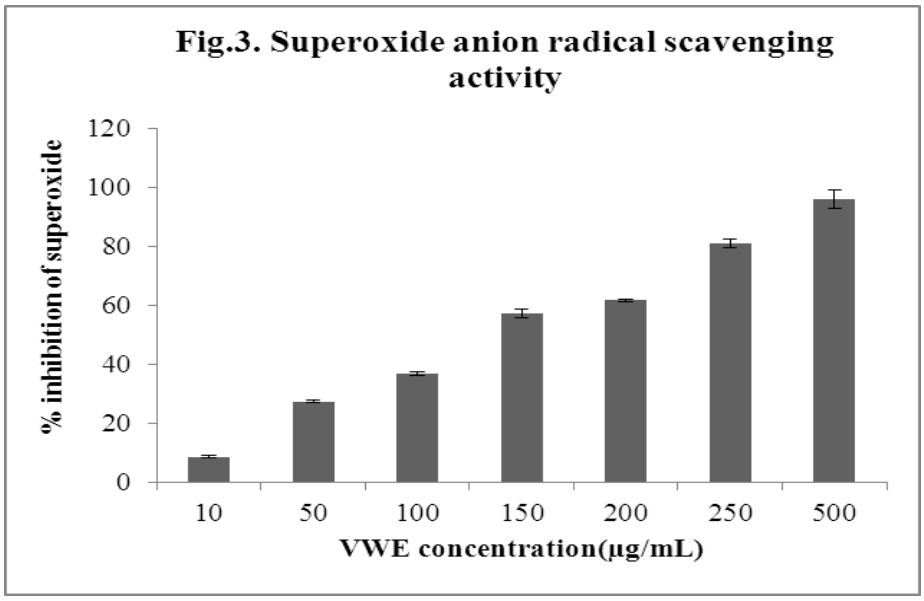

Figure 3. Superoxide anion radical scavenging activity of different concentrations of VWE

Values are expressed as mean ± SE of three independent experiments.

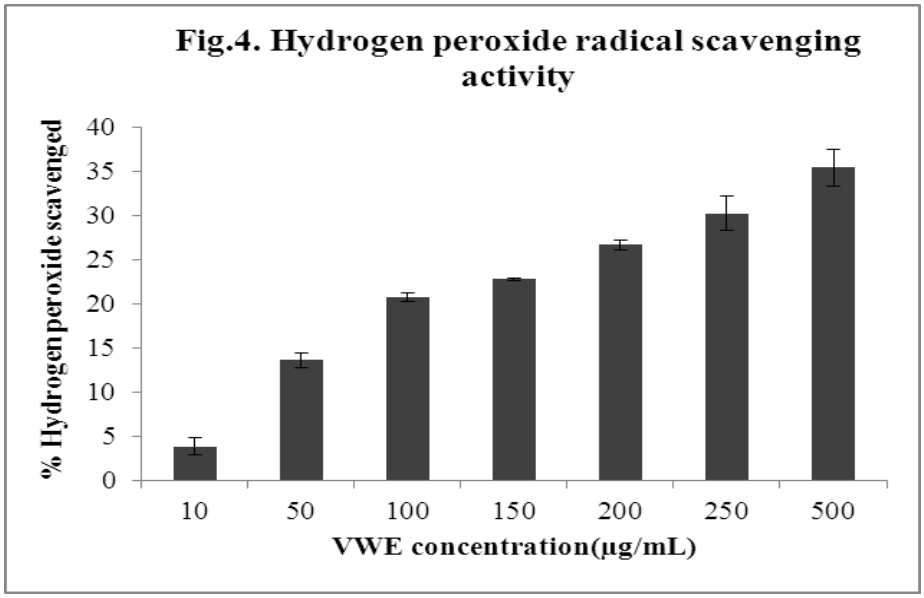

Figure 4. H_2O_2 scavenging activity of different concentrations of VWE

Values are expressed as mean ± SE of three independent experiments.

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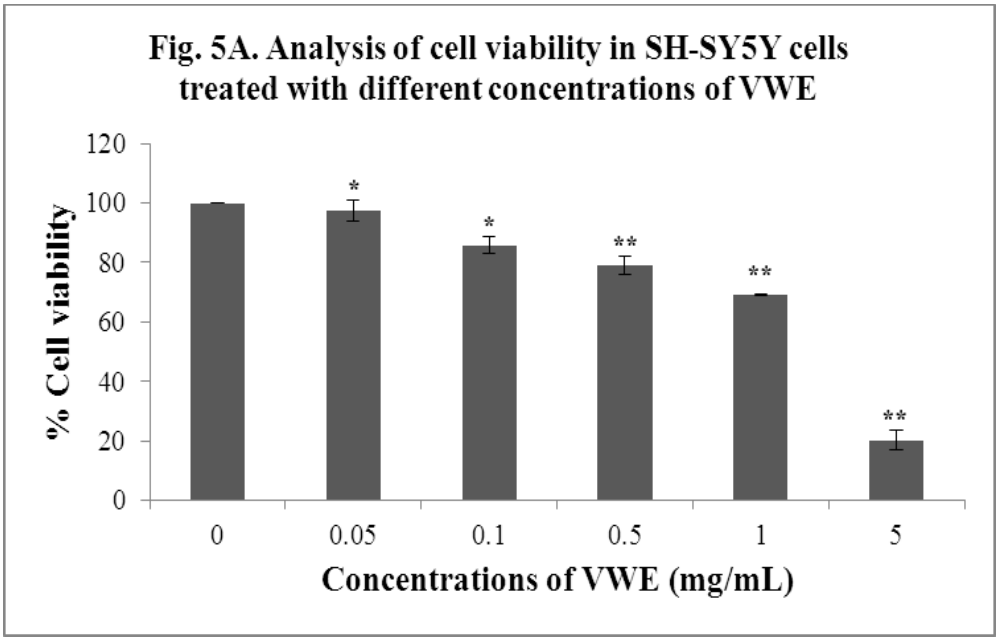

Figure 5A. Analysis of cell viability in SH-SY5Y cells treated with different concentrations of VWE

The cells were grown in 96 well plates and treated with 0.05, 0.1, 0.5, 1 and 5mg/mL concentrations of VWE for 24h. Cell viability was measured by MTT method. Values are expressed as mean ± SE of three independent experiments. One way ANOVA followed by Tukey's method. Values with superscript * differ significantly at $p \leq 0.05$ and the values with ** differ significantly at $p \leq 0.001$, as compared to untreated cells.

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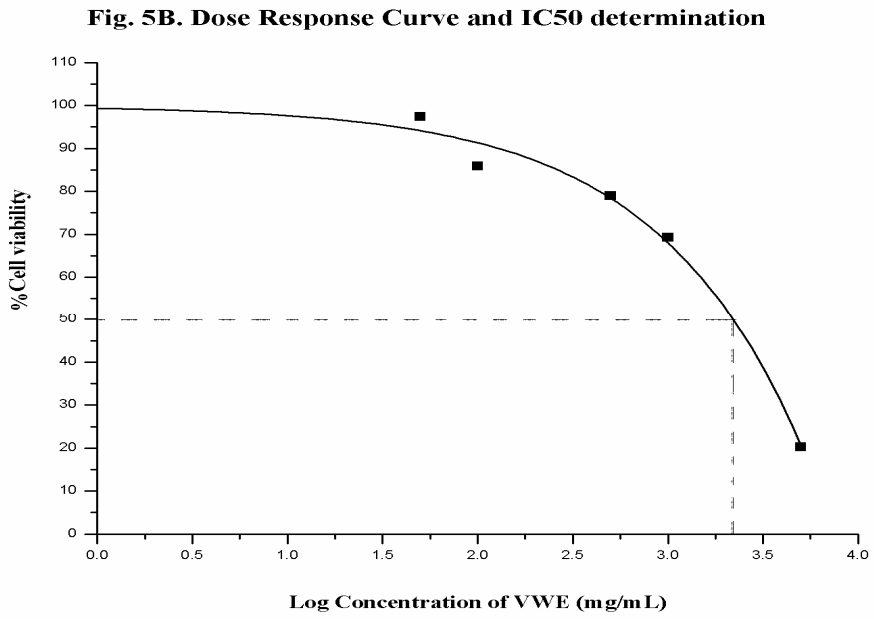

Figure 5B. Dose Response Curve and IC_{50} determination

Dose response curve of VWE was done by Non-linear curve fitting using Origin Pro 8 software. The concentrations of VWE were expressed as Log values in X axis and plotted against % cell viability. IC_{50} value was calculated by using the Finding X from Y tool in the software.

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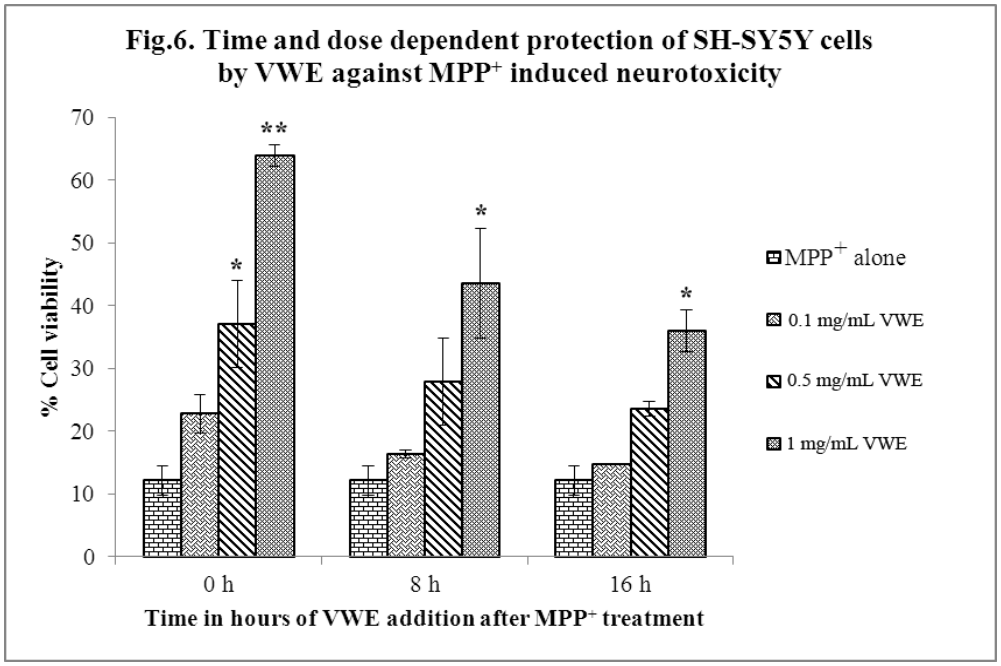

Figure 6. Time and dose dependent protection of SH-SY5Y cells by VWE against MPP^+ induced neurotoxicity

The cells were grown in 96 well plates and treated with 5mM of MPP^+. Varying concentrations of VWE (0.1, 0.5 and 1mg/mL) were added at 0h, 8h and 16h post MPP^+ treatment. The cell viability was measured after 24h by MTT method. Values are expressed as mean ± SE of three independent experiments. One way ANOVA followed by Tukey's method. Values with superscript * differ significantly at $p \leq 0.05$ and the values with ** differ significantly at $p \leq 0.001$, as compared to untreated cells.

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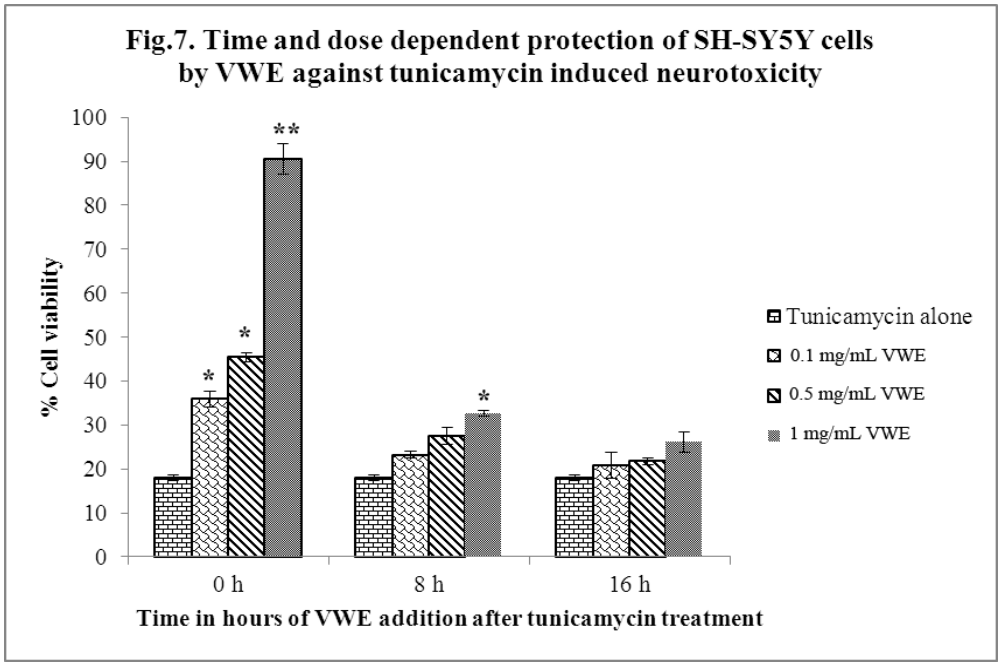

Figure 7. Time and dose dependent protection of SH-SY5Y cells by VWE against tunicamycin induced neurotoxicity

The cells were grown in 96 well plates and treated with 10µM of tunicamycin. Varying concentrations of VWE (0.1, 0.5 and 1mg/mL) were added at 0h, 8h and 16h post tunicamycin treatment. The cell viability was measured after 24h by MTT method. Values are expressed as mean ± SE of three independent experiments. One way ANOVA followed by Tukey's method. Values with superscript * differ significantly at $p \leq 0.05$ and the values with ** differ significantly at $p \leq 0.001$, as compared to untreated cells.

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Fig.8A. Morphological analysis of protective effect of VWE in SH-SY5Y cells against MPP^+

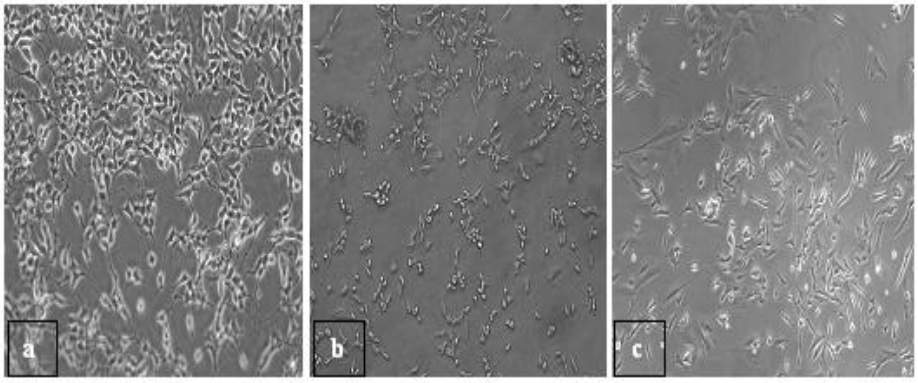

Fig.8B. Morphological analysis of protective effect of VWE in SH-SY5Y cells against and tunicamycin treated cells.

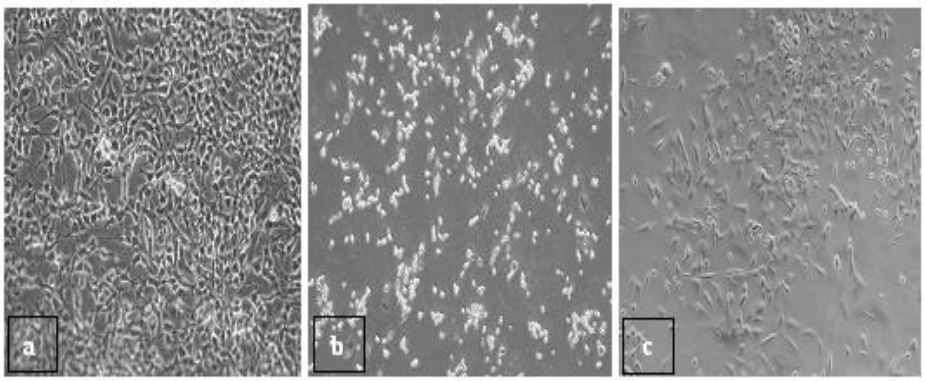

Figure 8. Morphological analysis of protective effect of VWE in SH-SY5Y cells against MPP^+ and tunicamycin treated cells

8A. Cells were treated with 5mM MPP^+. For analysing the protective effect of VWE 1mg/mL of the extract was added along with the toxin. Morphological changes were analysed after 24h with phase contrast microscope and pictures were taken at 200X. a- untreated control cells; b- cells treated with 5mM MPP^+ and c- cells treated with MPP^+ and VWE. **8B.** Cells were treated with 10µM tunicamycin. For analysing the protective effect of VWE, 1mg/mL of the extract was added along with tunicamycin. Morphological changes were analysed after 24h with phase contrast microscope and pictures were taken at 200X. a- untreated control cells; b- cells treated with 10µM tunicamycin and c- cells treated with 10µM tunicamycin and VWE.