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EVALUATION OF NEUROPROTECTIVE EFFECTS OF WEDELOLACTONE AGAINST GLUTAMATE-INDUCED NEUROTOXICITY IN PRIMARY RAT CORTEX NEURONAL CULTURE

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ABSTRACT

Glutamate excitotoxicity is one of the major pathogenesis behind the development and progression of neurodegenerative diseases. In the present study, we have evaluated the neuroprotective activity of wedelolactone (WL) against glutamate-induced neurotoxicity in primary rat cortex neurons (RCN). Treated the RCNs with WL 25 & 50 µg/ml for 2h and later treated the cells with 100µM glutamate (GLU) and incubated for 24h at 37ºC. Significant protection was observed with WL in a dose-dependent manner. The results demonstrated that WL could improve the antioxidant status in the neurons and inhibit proinflammatory cytokine production. The WL also maintained the Ca^{2+} homeostasis, IGF-1 expression, and protected the neurons from glutamate-induced neuronal toxicity. The neuroprotective activity of WL was further confirmed by the results of the NAA and MAP-2 expression. The results propose that the WL is significantly effective against glutamate-induced neurotoxicity and can protect neurons from various chemical events that are involved in the pathogenesis of neurotoxicity.

KEYWORDS: Wedelolactone; Glutamate excitotoxicity; Neurodegenerative diseases; Neurotoxicity; Neuronal protection.

1. INTRODUCTION

Glutamate excitotoxicity is the one of the major pathogenesis behind the development and progression of the motor neuron diseases such as sporadic amyotrophic lateral sclerosis (sALS). ALS is a life-threatening multisystemic multifaceted progressive neurodegenerative disease. The progression of the disease leads to the paralysis of the patient and death after 3-5 years after the onset of symptoms due to respiratory arrest. The multiple pathogeneses behind the development and progression of ALS make a challenge to find out a novel therapy for this disease. The factors behind the neurodegenerative disease are genetic, oxidative stress, glutamate excitotoxicity, protein aggregation, apoptosis, autoimmunity, production of inflammatory cytokines, alteration in a cellular process, metabolic impairment, mitochondrial damage, glial activation, and disruption in axonal transport.[1,2,3] Several studies supported the evidence of glutamate excitotoxicity in the pathogenesis of sALS. Hence, targeting glutamate excitotoxicity pathogenesis will help to bring up with therapeutic strategies against neurodegenerative diseases. The Riluzole (2-amino-6-trifluoromethoxy benzothiazole) is the first FDA approved drug for ALS condition and which acts via inhibiting kainate and NMDA (N-Methyl-D-aspartic acid) receptors.[4,5] Riluzole reduces glutamate release from nerve terminals; NMDA mediated effects and partially prevents *in vitro* neuronal degeneration produced by cerebrospinal fluid (CSF) of ALS Patients.[6,7] Another drug that FDA approved was Edaravone, (MCI-186, 3-methyl-1-phenyl-2-pyrazoline-5-one), is a free radical scavenger mainly used for the treatment of acute cerebral infarction. It can able to eliminate lipid peroxides and hydroxyl radicals and can protect from degeneration and oxidative stress. Studies found that progression of motor dysfunction was slowed in the Edaravone treated patients.[8,9] Both reduced the mortality and slowed the progression of ALS and extended the survival for a few months. However, the complete cure and the risks were negligible.

This study focused on glutamate-induced neurotoxicity, as it is the main pathogenesis behind sALS. During excitotoxicity, there will be an influx of Na^+ & Cl^- and destabilisation of intracellular Ca^{2+} homeostasis that activates a cascade of biochemical events and causes neuronal toxicity or neuronal death. Also, the disturbances in neuronal energy status trigger glutamate-induced neurotoxicity.[10] Apart from these, glutamate excitotoxicity causes reactive oxygen species (ROS) and Nitric oxide synthase (NOS) production and results in oxidative and nitrosative stress. The oxidative stress further opens mitochondrial permeability transition pore

and produce more ROS and worsens mitochondrial damage.[11,12] Mitochondrial dysfunction can induce neuronal death by elevating intracellular calcium level, stimulating free radical generation, or releasing apoptogenic proteins.[13] Thus, understanding the pathways involved in glutamate excitotoxicity is one of the significant steps for the future clinical treatment of many neurodegenerative diseases.

In this study, we have evaluated the neuroprotective effects of wedelolactone (WL) on glutamate excitotoxicity by using primary neuronal cultures of neonatal rat cortex neurons. WL is a coumestan derivative, which is mainly obtained from *Eclipta alba* and *Wedelia calendulacea*. Several studies reported WL for its antioxidant activity,[14] anti-inflammatory activity,[15] and anti-apoptotic activity.[16] Similarly, *Wedelia calendulacea* was reported for its protective effects on neuropharmacological actions.[17] The studies support the protective effects of WL and hence, we evaluated the impact of WL against glutamate-induced neurotoxicity via various mechanisms. We have performed cytotoxicity study for WL by MTT assay and determined the effect of WL on antioxidant status, proinflammatory cytokine production, calcium binding protein, insulin growth factor-1 (IGF-1), and maintaining neuronal plasticity. The purpose of this study is to understand the mechanism behind glutamate-induced neurotoxicity and the neuroprotective activity of WL against glutamate-induced toxicity.

2. Experimental procedures

Primary rat cortex neurons were collected from (Gibco-Life Technologies, USA) and we followed the methods described by Life technologies. The primary rat cortex neurons (RCN) were isolated from day-18 Fisher 344 rat embryos.

2.1. Media preparation

The medium and supplements were procured from Life Technologies, USA. Used Neurobasal medium for primary rat cortex neuronal culture. Added final concentration of 0.5mM GlutaMAX-1 supplement and 2% (v/v) of B-27 supplement to the Neurobasal medium before use.

2.2. Recovery and culture of RCN cells

Recovery and culture were performed as per the procedures described by Life Technologies, USA. Thawed the frozen vial by gently swirling it in a 37ºC water bath. The vial was disinfected with 70% isopropyl alcohol. Rinsed the Neurobasal/B-27 medium using a pipette and smoothly transferred the cells. Slowly added Neurobasal/B-27 medium to the tube (total suspension volume of 4 ml). To a microcentrifuge tube containing 0.4% trypan blue, added cell suspension and mixed by gently tapping. Determined the viable cell density using hemocytometer. Plated ~1 x 10^5 live cells per well in a poly-D-lysine-coated 48-well plate. Diluted the cell suspension by adding complete Neurobasal/B-27 medium. Incubated the cells at 36ºC-38ºC in a humidified atmosphere of 5% CO_2 in the air. After 24 hours of incubation, aspirated the half of the medium from each cell and replaced with fresh medium. Changed the medium every third day by aspirating half of the medium from each well and replacing it with fresh medium. The stock cultures then grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well microtiter plates (Tarsons India Pvt. Ltd., Kolkata).

2.3. Cytotoxicity study by MTT assay

The monolayer cell culture was trypsinised, and the cell count was adjusted to 1.0 x 10^5 cells/ml using the Neurobasal medium. To each well of the 96 well microtiter plate, the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and washed the monolayer once with medium and added 100 µl of different test concentrations (7.8, 15.6, 62.5, 125, 250, 500, and 1000 µg/ml) of WL (Baoji Guokang Biotechnology Co., Ltd., China; Purity-99.04%) on to the partial monolayer in microtiter plates. The plates were then incubated at 37ºC for three days in 5% CO_2 atmosphere, and microscopic examination was carried out. After 72h, the drug solutions in the wells were discarded, and MTT in PBS was added to each well. The plates were incubated for 3-hour at 37ºC in 5% CO_2 atmosphere. The supernatant was removed, and propanol was added, and the plates were gently shaken to solubilise the formed formazan. Measured the absorbance by using a microplate reader at a wavelength of 540 nm and calculated the percentage growth inhibition using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

2.4. Cell treatment

Cell treatment was don by adopting and modifying the procedure mentioned by Favaron et al., (1988).[18] Aspirated the medium and RCN cells were pre-incubated 2h at 37ºC with 25 & 50 µg/ml of WL before glutamate exposure. Treated the cells with 100µM glutamate (GLU) and incubated the cells for 24h at 37ºC. At the end of the treatment, trypsinised the cells and harvested the cells directly into 12 x 75 mm polystyrene tubes. Centrifuged the tubes at 300 x g at 25ºC. Decanted the supernatant and washed with PBS and suspended the pellet with a cell lysis solution per 1-5 x 10^6 cells. Centrifuged the suspension for 5 minutes at 12,000-14,000 x g at 2-8ºC and stored the supernatant at -80ºC for assays.

2.5. Estimation of reduced glutathione (GSH)

GSH activity was assayed according to the method of Ellman, (1959).[19] To the different cell supernatant, 0.02 M EDTA solution was added and kept on ice bath for 10 min. Then distilled water and of 50% (w/v) TCA was added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 g. To the supernatant, Tris

buffer (0.4M) was added. Then DTNB solution (Ellman's reagent; 0.01M DTNB in methanol) was added and vortexed thoroughly. Optical density (OD) was read (within 2-3 min after the addition of DTNB) at 415 nm in the spectrophotometer against a reagent blank.

2.6. Estimation of nitric oxide (NO)

This estimation was carries out by adopting the method from Kropf et al., (2010).[20] RCN cells were treated with test substances as described above and incubated for 24 h and conditioned media collected were used for nitrite determination. Determination of nitrite as a biomarker for NO was carried out. In brief, an equal volume of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride prepared in water, 1% sulfanilamide prepared in 5% phosphoric acid and cell culture media were mixed in flat bottom 96-well plate incubated for 10-15 min. Coloured end-product was measured at 530 nm. Percentage of nitric oxide inhibition was calculated over control.

2.7. Estimation of superoxide dismutase (SOD)

The Tris buffer and EDTA added to the cell lysate. The spectrometer was set at 420 nm, 0.3 ml of pyrogallol solution was added and then increase in absorbance and change in absorbance per minute was recorded against blank for three minutes at an interval of 30 seconds. The results were expressed as SOD activity U/mg protein. This procedure was performed as per Marklund and Marklund, (1974).[21]

2.8. Estimation of catalase

The estimation was performed by adopting the method from Aebi, (1974).[22] To the cell supernatant added of phosphate buffer of pH 7.0. The spectrometer was set at 240 nm. Added H_2O_2 and then recorded the absorbance against blank for 60 seconds at an interval of 15 seconds at 240 nm. The results were expressed as catalase activity U/mg protein.

2.9. Estimation of TNF-α in cell supernatant by bioassay

To each well of the 96 well microtiter plate, diluted cell suspension was added. After 24 h, when the partial monolayer was formed, the supernatant was flicked off, washed the monolayer, and 100 µl of diluted samples was added to the cells in quadruplicate wells. The cultures were then incubated at 37ºC for 24 h in 5% CO_2 atmosphere. After 24h, the drug solutions in the wells were discarded, and MTT in phosphate buffered saline (PBS) was added to each well. The plates were gently shaken and incubated for 3h at 37ºC in 5% CO_2 atmosphere. The supernatant was removed, and propanol was added, and the plates were gently shaken to solubilise the formed formazan. Measured the absorbance at a wavelength of 540 nm, and the percentage of cell viability was calculated. The cell viability is the direct indication of TNF-α production properties in RCN cells.

2.10. N-acetylaspartate (NAA) determination by HPLC

Prepared the mobile phase by mixing acetonitrile and ammonium acetate buffer having pH 2.9 (60:40). The standard solution was prepared by dissolving N-acetyl-aspartic acid with acetonitrile. Working solution with concentration 100 µg/ml was prepared by using stock solution with methanol. Sample preparation was performed by adding a test sample in acetonitrile. Then the solution was vortexed, centrifuged, and separated the supernatant. To the dried sample of acetonitrile was added and the sample was injected into the HPLC system. Calculated the concentration of the unknown sample and expressed as µg/ml.

2.11. Estimation of IL-6, IL-β, and IGF-1 by ELISA

The gene expression determined quantitatively by using rat IL-6, IL-β ELISA kits (Krishgen Biosystems, India) and rat IGF-1 ELISA kit (KINESISDx, USA). A monoclonal antibody specific for the gene has been coated onto the wells of the microtiter strips provided. Samples, including standards of known gene concentrations, control specimens or unknowns are pipetted into these wells. During the first incubation, the standards or samples and a biotinylated monoclonal antibody specific for a gene are simultaneously incubated. After washing, the enzyme Streptavidin-HRP, that binds the biotinylated antibody was added, incubated, and washed. A TMB substrate solution was added which acts on the bound enzyme to induce a coloured reaction product. The intensity of this coloured product was directly proportional to the concentration of specific gene present in the samples.

2.12. Calbindin and MAP-2 expression analysis by Reverse transcriptase-polymerase chain reaction transcriptase-polymerase chain reaction

The mRNA expression level of calbindin and microtubule associated protein (MAP-2) was carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the RCN (Rat Cortex Neuron) cells were cultured in 60 mm petridish and maintained in the specific medium for 48 hrs. The medium was supplemented with FBS and amphotericin. To the dish required concentration of test, samples were added and incubated for 24 hr. Total cellular RNA was isolated from the untreated (control) and treated cells using Tri Reagent according to manufacturer's protocol. cDNA was synthesised from total isolated RNA by reverse transcriptase kit according to manufacturer's instructions (Thermo scientific). Then the reaction mixture was subjected to PCR for amplification. cDNAs using specially designed primers procured from Eurofins India and as an internal control the housekeeping gene GAPDH was coamplified with each reaction. The specific primers for Calbindin and MAP-2 were acquired from Eurofins, India. The forward primer used for Calbindin amplification was 5' GATGCCAGCAACTGA AGT 3', and the reverse primer used was 5' GGCCTAAGCATAGA CTTT 3'. The forward primer

used for MAP-2 amplification was 5' CTGGCCTTTTTGGTTCTCAT 3', and the reverse primer used was 5' TAGTCTAAGCTTAGC 3'. After the amplification, the data was transferred, and fold amplification was calculated using Light cycler 96 analysis software.

2.13. Statistics

The p-value of less than 0.05 considered being statistically significant. Data are presented as mean ± Standard error mean (SEM), and data are shown as mean ± Standard error mean (SEM) and 'n' was taken as per statistical requirement. The software used for the analysis was GraphPad Prism version 5.01. Statistical analysis was performed by using one-way ANOVA followed by Tukey's post hoc test.

3. RESULTS

Conducted glutamate-induced neurotoxicity in the primary rat cortex neurons and observed the effects of wedelolactone (WL) against glutamate-induced toxicity. Cytotoxicity study was performed by MTT assay and determined CTC_{50} for WL, and the CTC_{50} was found to be 166.02 ± 4.36 µg/ml (Fig.1). The lower and higher doses selected for WL are 25 and 50 µg/ml.

Further studies were conducted to evaluate the protective effects of WL on the glutamate-induced neurotoxicity. The antioxidant activity was determined by estimating the amount of reduced glutathione (GSH) (Fig.2A), nitric oxide (Fig.2B), SOD (Fig.2C), and catalase activity (Fig.2D). We observed that both WL significantly increased the level of GSH [***p < 0.001, Df 11(3,8)], SOD[***p < 0.001, Df 11(3,8)], and catalase [***p < 0.001, Df 11(3,8)] in the cortex neurons. Similarly, WL significantly reduced the level of nitric oxide concentration [***p < 0.001, Df 11(3,8)]. Observed a decline in the antioxidant profile after the administration of glutamate. Among the doses, the higher dose (50 µg/ml) produced a better effect than 25 µg/ml treatment.

To confirm the further mechanism behind the protective effects of WL, we estimated the percentage inhibition of TNF-α (Fig.3A), and the expression of other cytokines such as IL-β (Fig.3B),, and IL-6 (Fig.3C). We observed that after the glutamate induction resulted in the production of cytokines and upregulated the expression of cytokines in the positive control group and WL significantly inhibit the TNF-α [***p < 0.001, Df 8(2,6)] and downregulated the expression of IL-β [***p < 0.001, Df 11(3,8)], and IL-6 [***p < 0.001, Df 11(3,8)]. Similarly, we have performed ELISA for IGF-1 (Fig.3D) expression and observed a significant upregulation of IGF-1 [***p < 0.001, Df 11(3,8)] in the WL (25 µg/ml & 50 µg/ml) treated groups. The IGF-1 expression was down-regulated in the glutamate-induced positive control group.

Similarly, we have determined the expression of calbindin (Fig.4A) in cortex neuron after glutamate toxicity by RT-PCR. The results produced a significant upregulation in the calbindin expression [***p < 0.001, Df 8(2,6)] in the WL treated groups and significant downregulation of calbindin in the positive control group. The estimation of NAA (Fig.4B) was performed to assess the neuronal damage and observed that NAA [***p < 0.001, Df 8(2,6)] level was declined in the positive control group. The treatment with WL produced a significant elevation in NAA level in the cortex neurons when compared with positive control. Also observed the expression of MAP-2, a neuron-specific marker (Fig.4C) and found downregulation of MAP-2 expression [***p < 0.001, Df 11(3,8)] in the positive control group. However, the treatment with WL significantly upregulated the MAP-2 expression in the cortex neurons.

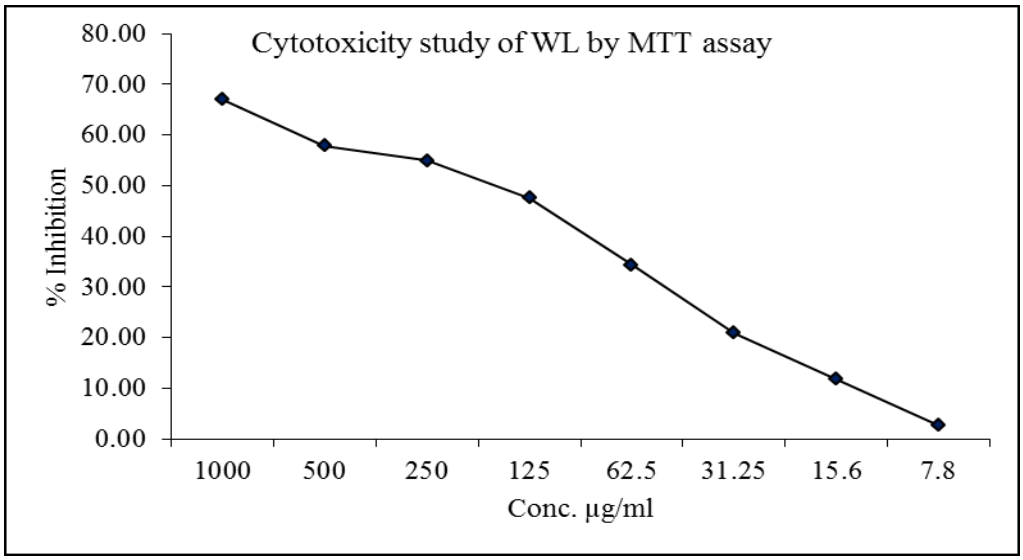

Fig. 1: Cytotoxic property of WL by MTT assay. The lower and higher doses of WL selected for *in vitro* study are 25 and 50 µg/ml.

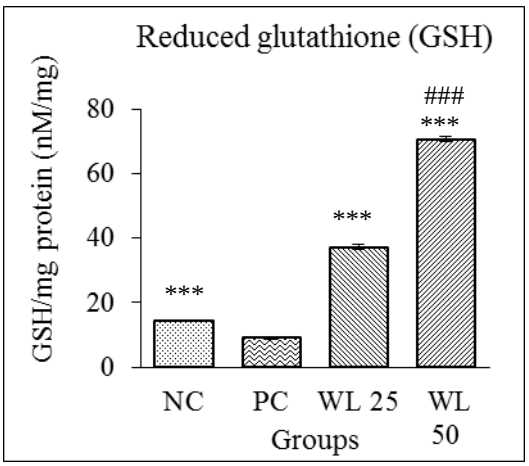

Fig.2A

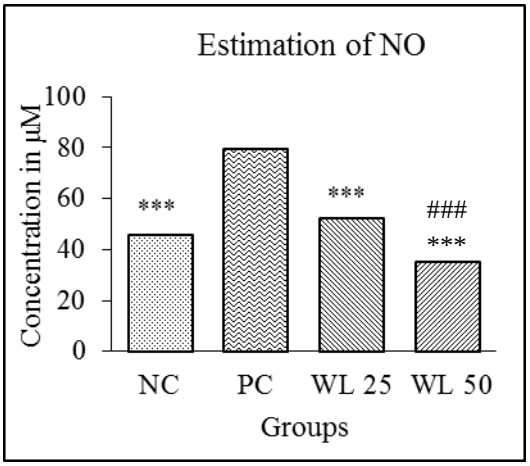

Fig.2B

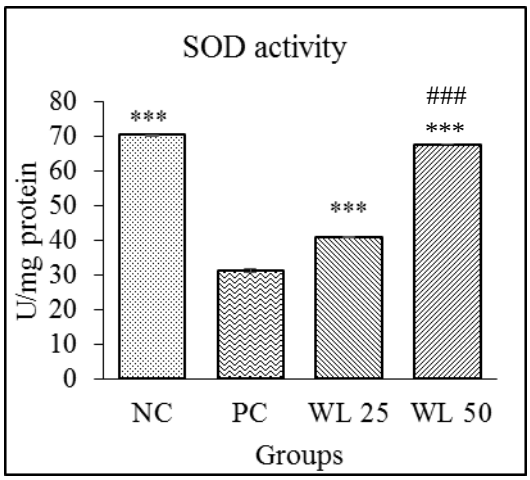

Fig.2C

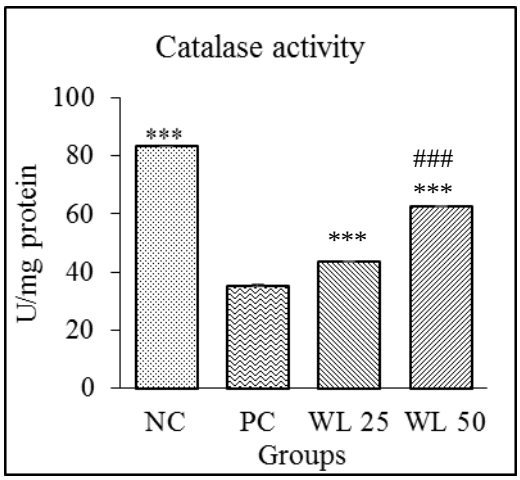

Fig.2D

Fig. 2: Effect of WL on (A) GSH activity; (B) Nitric oxide production; (C) SOD activity; (D) Catalase activity. NC- Normal Control, PC- Positive control (GLU treated), WL 25- GLU + WL 25 µg/ml, WL 50- GLU + WL 50 µg/ml. *- comparison with PC, ###- comparison with WL 25. Where n=3, ***p < 0.001, Df 11(3,8).**

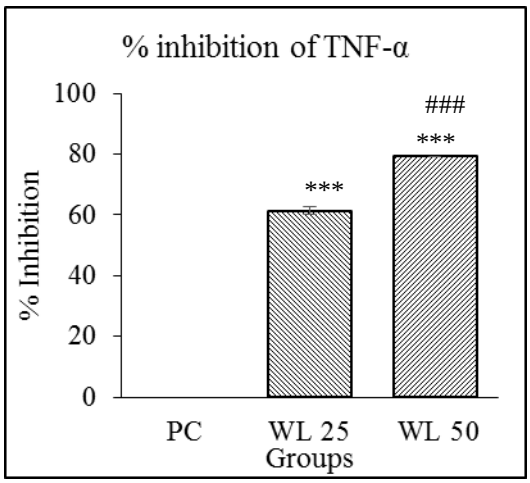

Fig.3A

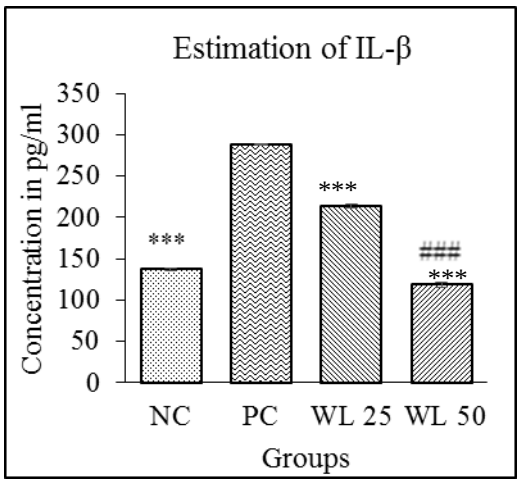

Fig.3B

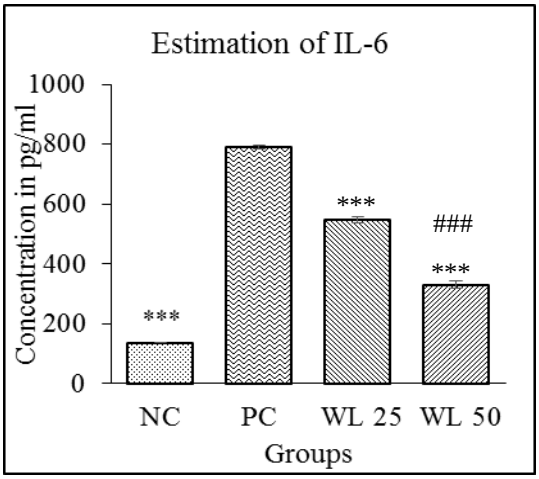

Fig.3C

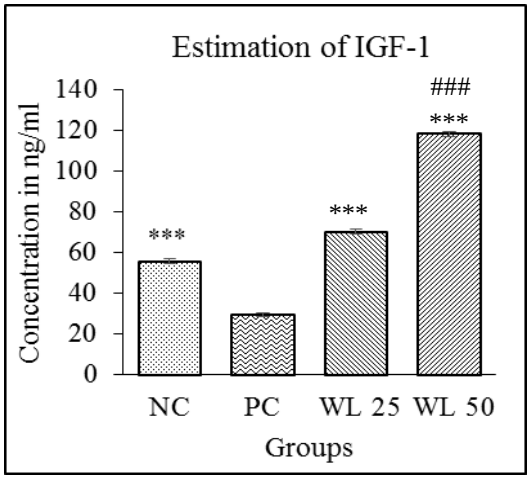

Fig.3D

Fig. 3: Effect of WL on (A) TNF-α inhibition, where ***p < 0.001, Df 8(2,6); (B) IL-β expression; (C) IL-6 expression; (D) IGF-1 expression. NC- Normal Control, PC- Positive control (GLU treated), WL 25- GLU + WL 25 µg/ml, WL 50- GLU + WL 50 µg/ml. *- comparison with PC, ###- comparison with WL 25. For Fig.3(B), 3(C), and 3(D) n=3, ***p < 0.001, Df 11(3,8).**

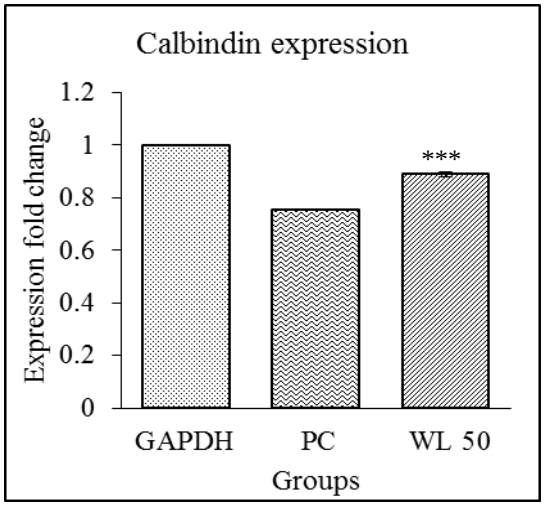

Fig.4A

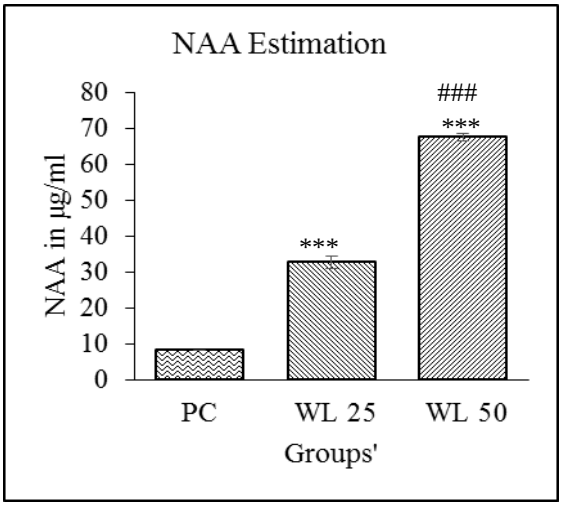

Fig.4B

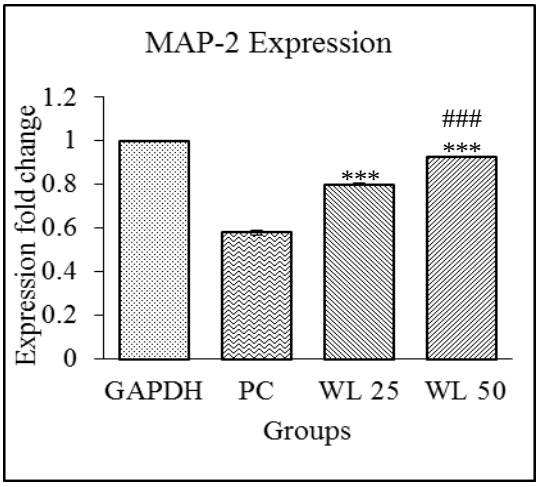

Fig.4C

Fig. 4: (A) Calbindin expression, where ***p < 0.001, Df 8(2,6); (B) NAA estimation, where ***p < 0.001, Df 8(2,6); (C) MAP-2 expression, where ***p < 0.001, Df 11(3,8). NC- Normal Control, PC- Positive control (GLU treated), WL 25- GLU + WL 25 µg/ml, WL 50- GLU + WL 50 µg/ml. *- comparison with PC, ###- comparison with WL 25. For Fig. 4(A), 4(B), and 4(C) n=3.**

4. DISCUSSION

Glutamate excitotoxicity is the one of the major hypothesis behind the development and the progression of the motor neuron diseases like sporadic ALS. Several studies claim the excess level of glutamate in the ALS patients.[23] Studies showed the elevated level of glutamate and related toxicity events mediated through AMPA receptor activation,[24] downregulation of glial glutamate-transporter EAAT2,[25] decreased glutamate uptake, and altered glutamine synthetase.[26] Similarly, NMDA receptor-mediated glutamate excitotoxicity was reported in chick embryo organotypic slice cultures.[27] Altogether, the evidence supports the glutamate excitotoxicity as a significant factor for neurodegeneration in ALS, Alzheimer's disease,[28] and Huntington's disease.[29] Hence, the therapeutic interventions, which can restore the glutamate level or protect the neurons from the glutamate-induced neurotoxicity, could stop the progression or development of several neurodegenerative diseases.

In the model of glutamate-induced neurotoxicity in primary neuronal cultures of neonatal rat cortex neurons, we observed that the WL significantly effective in protecting neurons from glutamate toxicity. The protective effect of WL through its antioxidant mechanism on glutamate-induced neurotoxicity was determined by estimating the level of GSH, nitric oxide, SOD, and catalase. Several studies proposed the accumulation of peroxides as a result of glutamate excitotoxicity and glutamate-induced reactive oxygen species generation and thereby alteration in the Ca^{2+} homeostasis.[30] The free radical formation will contribute to neuronal death in many neurodegenerative diseases. Involvement of NO production upon neuronal inflammation also proposed as a causative factor behind the reduced glutamate uptake and intracellular accumulation of glutamate.[31] In this study, we found that WL significantly effective in maintaining and increasing the oxidative status in the cortex neurons. WL increased the level of GSH, SOD, catalase, and decreased the level of NO production. The data indicate that, the excitotoxicity due to that excitotoxicity due to glutamate generates free radicals and causes neuronal death via excess accumulation of glutamate and alteration in the glutamatergic receptors.

The glutamate exposure results in the production of proinflammatory cytokines as a result of astroglial activation. The studies postulate that the proinflammatory mediators inhibit the astrocyte glutamate uptake and stimulate the nitric oxide production.[32] IL-6 is known to elevate Ca^{2+} level and which enhances NMDA receptor-mediated responses and neurotoxicity via stimulation of free radical formation.[33,34] The glutamate administration leads to the upregulation of proinflammatory cytokines in the rat cortex neurons. From the study proved that the WL is capable of inhibiting TNF-α and downregulating the proinflammatory cytokines (IL-1β, and IL-6) significantly in the rat cortex neurons.

The glutamate excitotoxicity resulted in the reduction of the IGF-1 level, and expression of calbindin in the neurons and triggered neuronal toxicity. IGF-1 is a key growth factor that regulates neurogenesis and synaptogenesis in the brain. The IGF-1 can inhibit NF-κB activation and can protect the neurons from inflammatory cascade via neuronal death. Also, it acts as an anti-apoptotic factor, and studies showed that IGF-1 could modulate Ca^{2+} homeostasis and promote survival of neurons.[35,36] Zheng et al., (2009) reported that glutamate inhibited the tyrosine phosphorylation of IGF-1 receptors and blocked the effects of IGF-1 on neuronal protection.[37] In this study, the results indicate that the glutamate excitotoxicity depleted the level of IGF-1 and prevented the protective effects of IGF-1 and caused neuronal damage. Similarly, the calbindin expression also affected by the glutamate toxicity. Calbindin is a cytosolic calcium-binding protein which are present in the brain, and which protect the neurons by modulating Ca^{2+} levels and synaptic plasticity.[38,39] From the study, we observed that suppression of calbindin expression in the glutamate-induced excitotoxicity neurons. The treatment with WL exhibited a significant effect against glutamate-induced neurotoxicity model by upregulating the IGF-1 and calbindin expression.

The neuroprotection was further confirmed by the results of NAA, MAP-2 expression in the cortex neurons. NAA is a neuron-specific metabolite and which helps to maintain neuronal plasticity. The concentration of NAA is very high in the brain of vertebrates and which is synthesised and stored in neurons.[40,41] Hence, the decline of the NAA level can be interpreted as the neuronal loss.[42] Similarly, MAP-2 is a neuron-specific protein that promotes assembly and stability of the microtubule network.[43] Several studies reported a decline in MAP-2 expression in neuronal inflammation and Ca^{2+} mediated neurotoxicity.[44,45] The glutamate toxicity depleted NAA and MAP-2 level in the neurons, and it indicates the presence of neuronal damage. However, the treatment with WL protected the neurons from neuronal loss due to glutamate toxicity, and the results show that the NAA and MAP-2 level were increased in WL treated group when compared to positive control.

Our results show that the glutamate-induced neurotoxicity resulted in the depletion of antioxidant status, alteration in the Ca^{2+} homeostasis, production of proinflammatory cytokines, reduction in the IGF-1 expression or dysregulation of the IGF-1 receptor, and neuronal damage via depleting the level of NAA and MAP-2. Glutamate induction causes ROS and NOS generation and which initiate the inflammatory cascade activation and mitochondrial damage. The depletion in the calbindin results in the increased intracellular Ca^{2+} and thereby glutamate excitotoxicity and neuronal

damage or neuronal death. The treatment with WL improved the antioxidant status in the neurons and found effective in inhibiting proinflammatory cytokine production. WL also maintained the Ca^{2+} homeostasis, IGF-1 expression, and protected the neurons from glutamate-induced neuronal toxicity. The overall results suggest that the WL is effective against glutamate-induced neurotoxicity.

5. CONCLUSION

The findings from our study propose that the glutamate-induced neurotoxicity is mediated through the free radicle formation, proinflammatory cytokine production, altered Ca^{2+} homeostasis, and declined IGF-1. Observed a down-regulation of MAP-2 expression and NAA concentration in the glutamate administrated cortex neurons and which indicate the presence of glutamate-induced neurotoxicity. The treatment with WL protected the cortex neurons from the glutamate-induced neurotoxicity and maintained the neuronal health. Further studies are needed to find out the primary pathways by which WL protects the neurons from the glutamate excitotoxicity. These findings might be a breakthrough in finding a therapy against several neurodegenerative diseases.

ABBREVIATIONS

CSF – Cerebrospinal fluid; DTNB – 5,5-Dithio-bis-(2-nitrobenzoic acid); EDTA – Ethylenediaminetetra acetic acid; GAPDH – Glyceraldehyde 3-phosphate dehydrogenase; GLU – Glutamate; GSH – Reduced glutathione; HRP – Horseradish peroxide; IGF – Insulin Growth Factor; MAP2 – Microtubule associated protein 2; NAA – N-Acetyl aspartate; NMDA – N-Methyl-D-aspartic acid; NO – Nitric oxide; NOS – Nitric oxide synthase; PBS – Phosphate buffered saline; RCN – Rat cortex neurons; ROS – Reactive oxygen species; sALS – sporadic Amyotrophic Lateral Sclerosis; SOD – Super oxide dismutase; TMB – 3,3',5,5'-tetramethylbenzidine; WL – Wedelolactone.

REFERENCES

1. Cluskey S, Ramsden DB. Mechanisms of neurodegeneration in amyotrophic lateral sclerosis. Mol Pathol, 2001; 54(6): 386-392.
2. Jellinger KA. Cell death mechanisms in neurodegeneration. J Cell Mol Med, 2001; 5(1): 1-17.
3. Shaw P. Molecular and cellular pathways of neurodegeneration in motor neuron disease. J Neurol Neurosurg Psychiatry, 2005; 76: 1046-57.
4. Debono MW, Le Guern J, Canton T, Doble A, Pradier L. Inhibition by riluzole of electrophysiological responses mediated by rat kainate and NMDA receptors expressed in Xenopus oocytes. Eur J Pharmacol, 1993; 235(2-3): 283-89.
5. Gupta K, Hardingham GE, Chandran S. NMDA receptor-dependent glutamate excitotoxicity in human embryonic stem cell-derived neurons. Neurosci Lett, 2013; 543: 95-100.
6. Couratier P, Sindou P, Esclaire F, Louvel E, Hugon J. Neuroprotective effects of riluzole in ALS CSF toxicity. Neuroreport, 1994; 5(8): 1012-14.
7. Doble A. The pharmacology and mechanism of action of riluzole. Neurol, 1996; 47(6): S233-41.
8. Abe K, Itoyama Y, Sobue G, Tsuji S, Aoki M, Doyu M, Hamada C, Kondo K, Yoneoka T, Akimoto M, Yoshino H. Confirmatory double-blind, parallel-group, placebo- controlled study of efficacy and safety of edaravone (MCI-186) in amyotrophic lateral sclerosis patients. Amyotroph Lateral Scler Frontotemporal Degener, 2014; 15(7-8): 610-17.
9. Abe K, Yuki S, Kogure K. Strong attenuation of ischemic and post ischemic brain edema in rats by a novel free radical scavenger. Stroke, 1988; 19(4): 480-5.
10. Shaw PJ, Ince PG. Glutamate, excitotoxicity and amyotrophic lateral sclerosis. J. Neurol, 1997; 244(2): S3-S14.
11. Castilho RF, Ward WM, Nicholls DG. Oxidative Stress, Mitochondrial function, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J Neurochem, 2001; 72(4): 1394-1401.
12. Nguyen D, Alavi MV, Kim KY, Kang T, Scott RT, Noh YH, Lindsey JD, Wissinger B, Ellisman MH, Weinreb RN, Perkins GA, Ju WK. A new vicious cycle involving glutamate excitotoxicity, oxidative stress and mitochondrial dynamics. Cell Death Dis, 2011; 2(12): e240.
13. Dong X, Wang Y, Qin Z. Molecular mechanisms of excitotoxicity and their relevance to the pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin, 2009; 30(4): 379-87.
14. Li X, Wang T, Liu J, Liu Y, Zhang J, Lin J, Zhao Z, Chen D. Effect and mechanism of wedelolactone as antioxidant-coumestan on ·OH-treated mesenchymal stem cells. Arab J Chem, 2017; http://doi.org/10.1016/j.arabjc.2017.03.008.
15. Yuan F, Jie C, Ping-Ping S, Guan S, Xu J. Wedelolactone inhibits LPS-induced pro-inflammation via NF-kappaB Pathway in RAW 264.7 cells. J Biomed Sci, 2013; 20(1): 1-11.
16. Lu Y, Hu D, Ma S, Zhao X, Wang S, Wei G, Wang X, Wen A, Wang A. Protective effect of wedelolactone against CCl4-induced acute liver injury in mice. Int immunopharmacol, 2016; 34: 44-52.
17. Prakash T, Rao NR, Viswanath Swamy AHM. Neuropharmacological studies on *Wedelia calendulacea* less stem extract. Phytomed, 2008; 15(11): 956-70.
18. Favaron M, Manev H, Alho H, Bertolino M, Ferret B, Guidotti A, Costa E. Gangliosides prevent glutamate and kainate neurotoxicity in primary neuronal cultures of neonatal rat cerebellum and cortex. Proc Natl Acad Sci USA, 1988; 85(19): 7351-5.
19. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys, 1959; 82: 70-77.

20. Kropf P, Kadolsky UD, Rogers M, Cloke TE, Muller I. The Leishmaniasis model. Methods Microbiol, 2010; 37: 307-28.
21. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem, 1974; 47(3): 469-74.
22. Aebi H. Methods of enzymatic analysis. 2nd ed., New York; Academic Press, 1974; 674.
23. Rothstein JD, Martin LJ, Kuncl RW. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. N Engl J Med, 1992; 326(22): 1464-8.
24. Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc Natl Acad Sci USA, 1993; 90(14): 6591-5.
25. Ferrarese C, Sala G, Riva R, Begni B, Zoia C, Tremolizzo L, Galimberti G, Millul, Bastone, Mennini T, Balzarini C, Frattola L, Beghi E. Decreased platelet glutamate uptake in patients with amyotrophic lateral sclerosis. Neurol, 2011; 56(2): 270-2.
26. Bos IWM, Hoogland G, Meine Jansen CF, Van Willigen G, Spierenburg HA, Van Den Berg LH, De Graan PNE. Increased glutamine synthetase but normal EAAT2 expression in platelets of ALS patients. Neurochem Int, 2006; 48(4): 306-11.
27. Brunet N, Tarabal O, Esquerda JE, Calderó J. Excitotoxic motoneuron degeneration induced by glutamate receptor agonists and mitochondrial toxins in organotypic cultures of chick embryo spinal cord. J Comp Neurol, 2009; 516(4): 277-90.
28. Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci, 1992; 12(2): 376-89.
29. Miller BR, Dorner JL, Shou M, Sari Y, Barton SJ, Sengelaub DR, Kennedy RT, Rebec GV. Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. Neurosci, 2008; 153(1): 329-37.
30. Mattson MP, Lovell MA, Furukawa K, Markesbery WR. Neurotrophic factors attenuate glutamate-induced accumulation of peroxides, elevation of intracellular Ca^{2+} concentration, and neurotoxicity and increase antioxidant enzyme activities in hippocampal neurons. J Neurochem, 1995; 65(4): 1740-51.
31. Weidinger A, Moldzio R, Toro G, Mkrtchyan G, Dumitrescu S, Trofimova L, Sole LVG, Duvigneau JC, Szabo C, Redl H, Bunik V, Koziov AV. 94-Glutamate excitotoxicity induced by nitric oxide mediated dysfunction of the mitochondrial 2-oxoglutarate dehydrogenase complex. Free Radic Biol Med, 2017; 112(1): 74.
32. Hu S, Sheng WS, Ehrlich LC, Peterson PK, Chao CC. Cytokine effects on glutamate uptake by human astrocytes. Neuroimmunomodulation, 2000; 7(3): 153-9.
33. Pober JS, Cotran RS. Cytokines and endothelial cell biology. Physio Rev, 1990; 70(2): 427-51.
34. Qiu Z, Sweeney DD, Netzeband JG, Gruol DL. Chronic interleukin-6 alters NMDA receptor mediated membrane responses and enhances neurotoxicity in developing CNS neurons. J Neurosci, 1998; 18(24): 10445-56.
35. Sun C, Meng Q, Zhang L, Wang H, Quirion R, Zheng W. Glutamate attenuates IGF-1 receptor tyrosine phosphorylation in mouse brain: Possible significance in ischemic brain damage. Neurosci Res, 2012; 74(3-4): 290-7.
36. Zhang F, Wang L, Liu Z, Xing Z, Wang H, Li Z. Insulin-like growth factor-1 modulates Ca^{2+} homeostasis and apoptosis of cultured dorsal root ganglion neurons with excitotoxicity induced by glutamate. Pharmazie, 2010; 65: 5-8.
37. Zheng WH, Quirion R. Glutamate acting on NMDA receptors attenuates IGF-1 receptor tyrosine phosphorylation and its survival signalling properties in rat hippocampal neurons. J Biol Chem, 2009; 284(2): 855-61.
38. Lukas W, Jones KA. Cortical neurons containing calretinin are selectively resistant to calcium overload and excitotoxicity *in vitro*. Neurosci, 1994; 61(2): 307-16.
39. Kook SY, Jeong H, Kang MJ, Park R, Shin HJ, Han SH, Son SM, Song H, Baik SH, Moon M, Yi EC, Hwang D, Mook-Jung I. Crucial role of calbindin-D_{28k} in the pathogenesis of Alzheimer's disease mouse model. Cell Death Differ, 2014; 21(10): 1575-87.
40. Baslow MH. N-acetylaspartate in the vertebrate brain: metabolism and function. Neurochem Res, 2003; 28(6): 941-53.
41. Tallan HH. Studies on the distribution of N-acetyl-L-aspartic acid in brain. J Biol Chem, 1957; 224(1): 41-5.
42. Clark JB. N-acetyl aspartate: A marker for neuronal loss or mitochondrial dysfunction. Dev. Neurosci, 1998; 20(4-5): 271-6.
43. Johnson GVM, Jope RS. The role of microtubule-associated protein 2 (MAP-2) in neuronal growth, plasticity, and generation. J Neurosci Res, 1992; 33(4): 505-12.
44. Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents *in vitro* glutamate-induced neuronal death. Neurosci, 1997; 76(1): 105-16.
45. Marx CE, Jarskog LF, Lauder JM, Lieberman JA, Gilmore JH. Cytokine effects on cortical neuron MAP-2 immunoreactivity: implications for schizophrenia. Biol Psychiatry, 2001; 50(10): 743-9.