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Der Pharmacia Lettre, 2020, 12 (9): 13-21
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Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Neuroprotective Activity of *Biophytum reinwardtii* on Hippocampal Neuronal HT22 Cells

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ABSTRACT

Background: *Glutamate is a major endogenous excitatory neurotransmitter in the brain and contributes to the development of neurodegenerative diseases by excessive activation.*

Purpose: *The purpose of the present study was to determine the neuroprotective effect methanolic extract of Biophytum reinwardtii (BR) against glutamate-induced oxidative stress and to assess the underlying mechanism.*

Method: *BR extract was subjected to a neuroprotective effect assay in HT22 mouse hippocampal cells. The mechanism underlying the neuroprotective effect of BR extract was evaluated by assaying reactive oxygen species (ROS) levels, intracellular Ca^{2+} levels, mitochondrial membrane potential, and glutathione level and antioxidant enzyme activity in HT22 cells.*

Results: *BR extract significantly decreased glutamate-induced death of HT22 cells (88.23 ± 1.65% relative neuroprotection). BR extract reduced the intracellular ROS and Ca^{2+} levels and increased the glutathione level and glutathione reductase and glutathione peroxide activities. Moreover, BR extract attenuated the mitochondrial*

membrane potential in HT22 cells. Conclusion: These results suggested that BR extract exerts a neuroprotective effect against oxidative stress HT22 cells, which was mediated by its antioxidant activity.

Keywords: Neuroprotective, Glutamate, Oxidative stress, Hippocampal cells, Antioxidant.

INTRODUCTION

Glutamate is a central nervous system (CNS) endogenous excitatory neurotransmitter. In memory, neuronal differentiation, and neural transmission, it plays important roles in synapse [1]. A high concentration of glutamate, however, contributes to a loss of learning and memory and is implicated in the pathogenesis of neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) [2]. One is the excitotoxic pathway regulated by glutamate receptors in ionotropic shape. The excitotoxicity process has been thoroughly characterized and transient Ca^{2+} influx is believed to be affected in alterations in calcium homeostasis, increases in the levels of reactive oxygen species (ROS), and ultimately cell death [3]. The second distinct pathway in glutamate toxicity does not include glutamate receptors, but rather a glutamate/cystine antiporter required to deliver cystine to neuronal cells [4]. High concentrations of extracellular glutamate inhibiting cystine absorption leads to an imbalance in cellular cysteine homeostasis, a reduction in cellular glutathione levels, and a reduction in cellular glutathione levels. In neuronal cell lines, primary neuronal cultures, and oligodendrocytes, glutamate-induced oxidative toxicity has been identified. This mechanism of oxidative neuronal death is thought to lead in many brain disorders to neuronal damage and degeneration [5, 6]. HT22 cells, an immortalized hippocampal cell line of the mouse, were widely used as an *in vitro* model to elucidate the mechanism of oxidative stress-induced neurotoxicity [7]. HT22 cells lack a functional ionotropic glutamate receptor, excluding excitotoxicity as a cause of glutamate-induced cell death. In Raw 264.7 murine macrophages, BR also has anti-inflammatory effects on lipopolysaccharide (LPS)-induced nitric oxide synthase (iNOS) and pro-inflammatory cytokines. It has not been confirmed, however, that BR is a neuroprotectant that can minimize apoptosis triggered by glutamate. We therefore looked at the protective effect of BR against glutamate-induced oxidative stress in a mouse hippocampal neuronal cell line.

Biophytum reinwardtii, also known as little tree plant, is a species of plant in the Oxalidaceae family and belongs to the genus *Biophytum*. It is commonly found in wet lands of Nepal, tropical India and in other Southeast Asian countries. Chemical analyses have shown that the plant parts are rich in compounds such as amentoflavone, cupressuflavone, and isoorientin. Its extracts are traditionally believed to be antibacterial, anti-inflammatory, antioxidant, antitumor, radioprotective, chemoprotective, antiangiogenetic, wound-healing, immunomodulatory, anti-diabetic, and cardioprotective in nature [8]. In present we focused on Neuroprotective effects of *Biophytum reinwardtii* methanolic extract in mouse hippocampal neuronal cells

MATERIALS AND METHODS

Plant materials

Biophytum reinwardtii whole plant was collected from forest area of Rampachowdavaram, East Godavari and authenticated by Dr. P. Prasanna Kumari Department of Botany, D.N.R College, Bhimavaram A voucher specimen was kept at Department of Pharmacology, Shri Vishnu College of Pharmacy.

Preparation of plant extract: The Whole plant were dried under shade at room temperature The shade dried, coarsely powdered roots (500 g) was successively extracted with petroleum ether (60-80oC) for 7 days to remove fatty matter. The defatted marc was then subjected to soxhlet extraction with 95% methanol to obtain methanolic extract. The methanolic extract was evaporated under reduced pressure at low temperature (30oC) to dryness and brownish yellow color extracts of *Biophytum reinwardtii* was obtained [9].

Cell culture: HT22 cells, a neuronal cell line derived from the mouse hippocampus, are used to study the mechanisms of glutamate-induced cell death. HT22 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin/streptomycin, NaHCO3 (2mg/mL), and 15 mM HEPES and were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability: Cell viability was determined by MTT assay as described previously [10]. Cultured HT22 cells were seed at 1.7×10^4 /well in 48-well plates and incubated for 24 h at 37°C in 5% CO_2. Cells were treated with 10, and 100 µg/ml BR extract and 50 µM Trolox (water-soluble analog of vitamin E sold: positive control) for 1 h, glutamate (2 mM) was added and cells incubated for 24h. After incubation, 1mg/mL MTT solution was added to each well for 3 h. MTT formazan was dissolved by dimethyl sulfoxide (DMSO) and the optical density at 570nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader.

Measurement of intracellular ROS levels: ROS formation induces neuronal cell death due to oxidative stress. Glutamate is involved in ROS production through NMDA receptor activation and intercellular Ca^{2+} accumulation. We evaluated ROS production using the dye 2′7′-dichlorofuorescein diacetate (DCF-DA) in HT22 cells. Sample BR extract (10 and 100 µg/ml), were treated with 2 mM glutamate for 8 h in the seeding cells. Subsequently, 10 µM DCF-DA was added to the cells, followed by incubation at 37°C for 30 min. DMEM was removed, cells washed twice with phosphate-bufered saline (PBS; 0.01 M, pH 7.4) and extracted with 1% Triton X-100 in PBS (0.01M, pH 7.4) for 10 min at 37°C. Fluorescence was measured at an excitation wavelength of 490 nm and emission wavelength of 525 nm for detection of ROS.

Measurement of Ca^{2+} levels: High concentrations of glutamate lead to intercellular Ca^{2+} accumulation due to activation of NMDA receptors. Increased Ca^{2+} levels have been implicated in neuronal cell death through depolarization of the mitochondrial membrane. The cytosolic Ca^{2+} concentration in HT22 cells was measured using Fura-2AM. HT22 cells were cultured at 37°C in 5% CO_2. HT22 cells were then treated with BR extract (10 and 100 µg/ml), 2 µM Fura-AM, and glutamate. After 20min, cells were washed with HEPES buffer saline and incubated for 1h. Fluorescence was monitored at an excitation wavelength of 380nm with fixed emission at 510 nm.

Measurement of mitochondrial membrane potential (MMP): MMP was measured using the fluorescent dye Rh123, as reported previously [11]. Rh123 was added to HT22 cells to a final concentration of 10 µM for 30 min at 37°C after the cells had been treated and washed with PBS. Fluorescence was monitored at an excitation wavelength of 488nm with fixed emission at 525nm.

Measurement of total glutathione content and antioxidant enzyme activities: Cells were seeded onto six-well plates at a density of 2×10^4 /ml and incubated for 24 h. Cells were treated with test compounds for 1 h, followed by 2 mM glutamate. After 24 h, cells were washed twice with PBS. The cell lysate was centrifuged for 30 min at 10,000 g at 4°C, and the supernatant was used for measurements of antioxidant activity and GSH contents.

Free-radical scavenging assay: A 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was conducted to evaluate antioxidant activity. Various concentrations of samples were added to 150 µL of 0.4 mM DPPH methanol solution in 96-well plates. T_e absorbance of DPPH solution at 517nm was measured using an ELISA reader. DPPH radical-scavenging activity was expressed and calculated as % inhibition=(1−As/Ao) × 100, where "As" is sample absorbance and "Ao" is absorbance of only DPPH solution.

Statistical analysis

All experiments were independently repeated a minimum of three times. All data are presented as mean ± SE. Statistical analyses were determined by using one-way ANOVA followed by Fisher's protected least significant difference test as a posthoc test for multiple comparisons. Values of $p<0.001$ were considered to be significant.

RESULTS

Neuroprotective effect of BR on glutamate-induced oxidative cytotoxicity

MTT assay was performed to investigate the neuroprotective effect of BR on glutamate-induced death of HT22 cells. We found that 2 mM glutamate treatment reduced cell viability. BR extract significantly improved the glutamate-mediated decrease in cell viability. BR extract (100 µg/ml) recovered cell viability to 88.23 ± 1.65% of the control, whereas the viability of the 2 mM glutamate-treated group decreased to 42.17 ± 5.11%. (Figure 1 and Figure 2) Trolox, as a positive control, also significantly protected HT22 cells against glutamate-induced cytotoxicity.

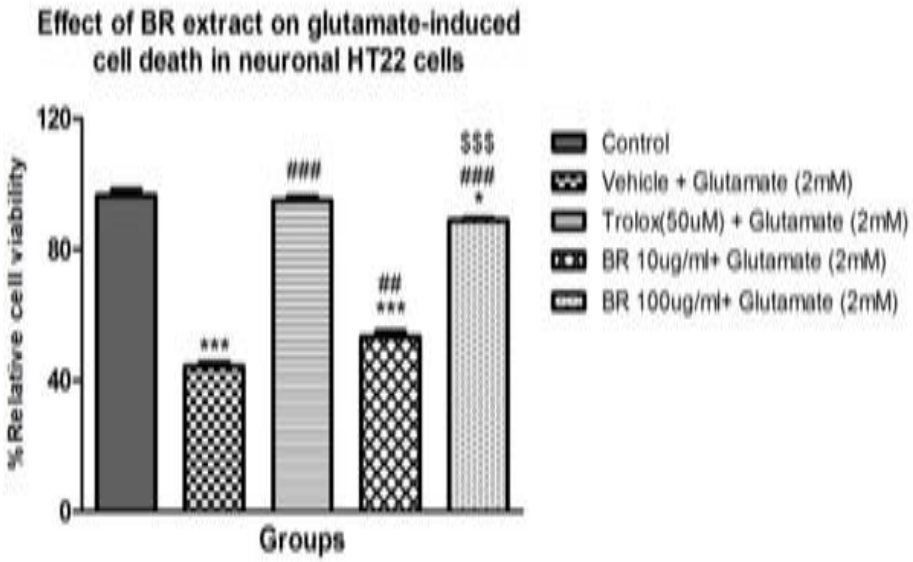

Figure 1: Effect of BR extract on glutamate induced cell death in hipocampal neuronal HT22 cells. Glutamate is decreased % relative cell viability when compared to normal, where as Tolox and BR increases % relative cell viability. Values are representing means of triplicate of experiment. ***$p<0.01$ significant than normal, ###$p<0.001$ more significant than glutamate treated cells.

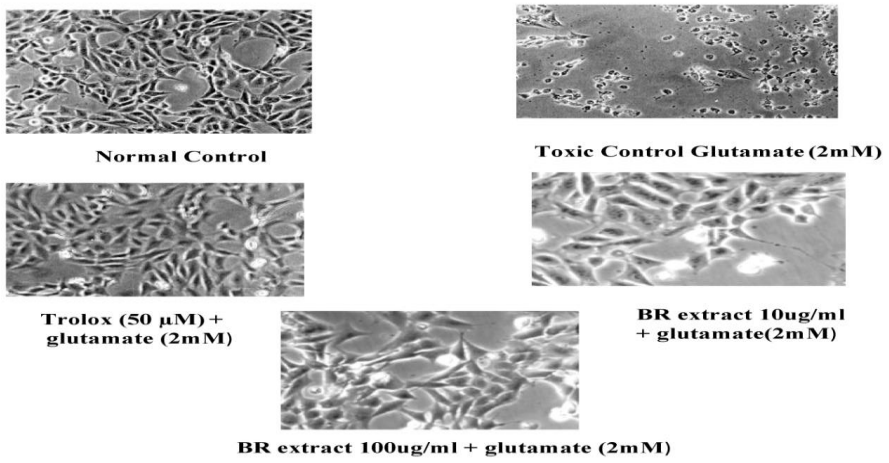

Figure 2: Neuroprotective effect of BR on glutamate-induced oxidative cytotoxicity.

Effect of MEBR on glutamate-induced ROS accumulation

Glutamate-treated cells exhibited increased fluorescence compared to the controls. BR extract pretreatment of cells inhibited ROS overproduction in a dose-dependent manner; 100 µg/ml BR extract significantly decreased ROS production to 120.18 ± 5.08% (Figure 3). Therefore, BR extracts protected HT22 cells against glutamate-induced death by inhibiting ROS production**.**

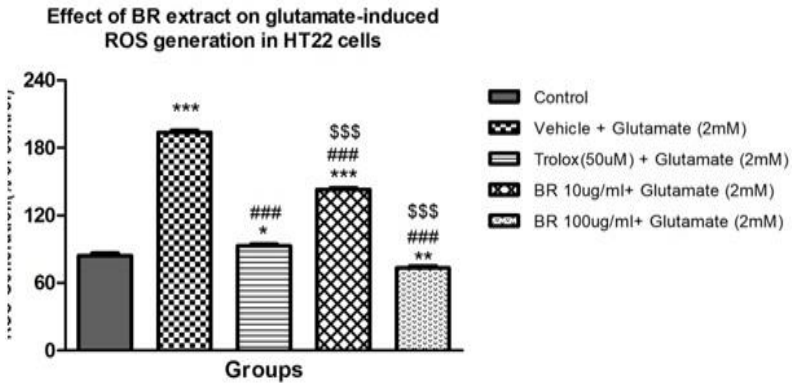

Figure 3: Effect of BR extract on glutamate induced ROS generation in hipocampal neuronal HT22 cells. Glutamate is increased % ROS generation when compared to normal, where as Tolox and BR decreases % ROS generation. Values are representing means of triplicate of experiment. ***p<0.01 significant than normal, ###p<0.001 more significant than glutamate treated cells.

Effect of MEBR on glutamate-induced Ca^{2+} influx

We evaluated the effect of BR on intracellular Ca^{2+} levels in HT22 cells using Fura-AM. The intracellular Ca^{2+} concentration was increased in cells treated with glutamate to 170.2 ± 2.56% (Figure 4). However, the Ca^{2+} concentration in cells pretreated with BR (100 µg/mL) decreased significantly compared with glutamate-treated cells (122.2 ± .05%).

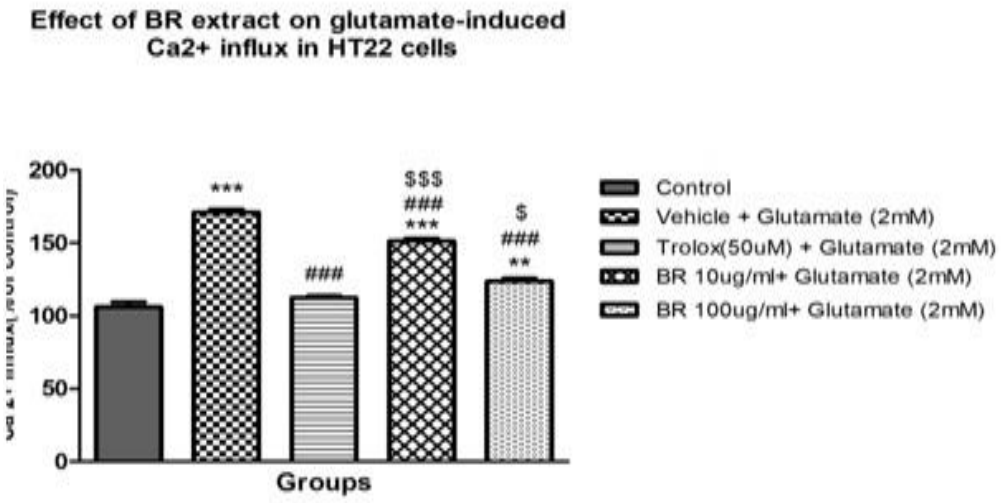

Figure 4: Effect of BR extract on glutamate induced Ca^{2+} influx in hipocampal neuronal HT22 cells. Glutamate is increased % Ca^{2+} influx when compared to normal, where as Tolox and BR decreases % Ca^{2+} influx .Values are representing means of triplicate of experiment .***p<0.01 significant than normal, ###p<0.001 more significant than glutamate treated cells.

Effect of MEBR on glutamate-induced mitochondrial dysfunction

To characterize the effects of Drp-1 on mitochondrial dysfunction induced by glutamate treatment, the MMP was monitored using the Rh123 probe, and the results indicated a glutamate-induced loss of MMP. ROS production causes mitochondrial injury and disrupts the MMP. Figure 5 shows that BR extracts significantly recovered the mitochondrial membrane potential to 119.2 ± 3.15% of the control at 100 µg/ml.

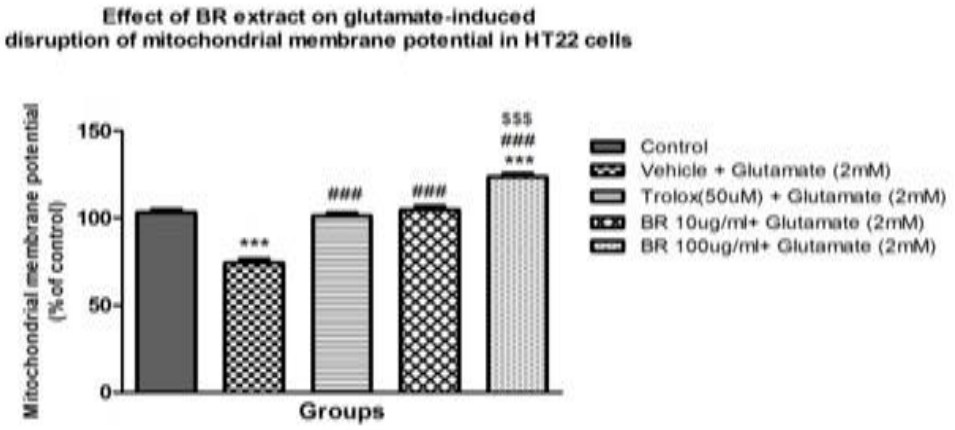

Figure 5: Effect of BR extract on glutamate induced disruption of mitochondrial membrane potential in hippocampal neuronal HT22 cells. Glutamate is decreased mitochondrial membrane potential when compared to normal, where as Tolox and BR increases mitochondrial membrane potential. Values are representing means of triplicate of experiment. ***p<0.01 significant than normal, ###p<0.001 more significant than glutamate treated cells.

Effect of MEBR on GSH, GR and GPx levels

We confirmed that glutamate-induced death of HT22 cells was related to oxidative stress and the treatment with BR recovered the oxidative stress condition. GSH is an important antioxidant in the CNS and glutathione reductase (GR) and glutathione peroxide (GPx) is a critical enzyme for the production of GSH. A high concentration of glutamate leads to deprivation of GSH by inhibiting cysteine uptake into cells. Depletion of GSH or antioxidant enzymes, such as GR and GPx, causes neuronal cell death. We investigated the effect of BR on GSH, GR and GPx expression levels were similar, as shown in Graph 5. The GSH, GPx and GR expression levels in glutamate-injured cells decreased to 30.18 ± 3.18%, 55.35 ± 8.25% and 62.82 ± 3.75%, respectively. However, BR extracts prevented the glutamate-induced depletion of GSH (58.21 ± 3.17% at100 µg/ml), GR (85.65 ± 2.76% at 100 µg/ml) and GPx (95.10 ± 1.60%) at 100 µg/ml) (Figure 6). These results suggest that BR exerted a neuroprotective effect, which was mediated by its antioxidant activity.

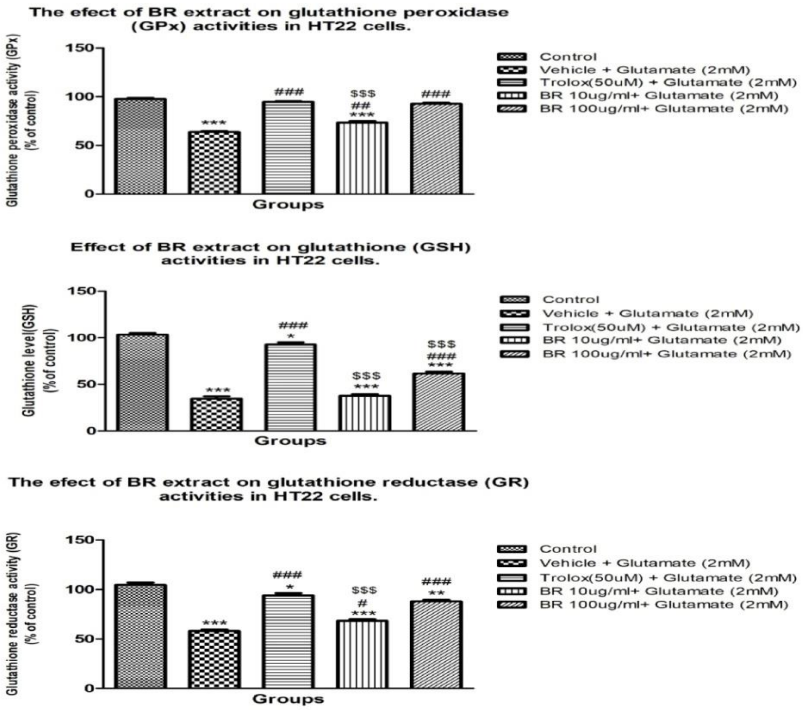

Figure 6: Effect of BR extract on GSH, GR and GPx activities in hipocampal neuronal HT22 cells. Glutamate is decreased GSH, GR and GPx activity when compared to normal, where as Tolox and BR increases GSH, GR and GPx activity. Values are representing means of triplicate of experiment. ***p<0.01 significant than normal, ###p<0.001 more significant than glutamate treated cells.

Antioxidant activity of BR on DPPH radical scavenging assay

DPPH radical-scavenging activity was investigated to determine the antioxidant activity of BR. Te DPPH radical-scavenging activity of BR extracts showed an IC50 value of 355.25 ug/ml. Positive control, trolox showed an IC50 value of 29.63µg/ml. This result that BR showed antioxidant effect, but lower effect than antioxidant effect of trolox

DISCUSSION

Oxidative stress elicited by glutamate induces both apoptotic and necrotic death of HT22 cells [12]. Glutamate-mediated neuronal cell death is closely associated with oxidative stress, including excessive reactive oxygen species production. Glutamate toxicity is induced mainly by glutamate receptor-mediated excitotoxicity and ROS-mediated oxidation in neuronal cells [13]. Pretreatment with methanolic extract of BR exerted protective effects, reducing neuronal death at higher concentration in HT22 cells according to our results. Antioxidant system imbalance induces Ca^{2+} influx, ROS accumulation, and lipid peroxidation. Ca^{2+} is an intracellular second messenger in the central nervous system [14]. Increased intracellular ROS levels due to massive Ca^{2+} influx disturb the activity of lipoxygenases (LOXs), and induce lipid peroxidation and mitochondrial dysfunction, resulting in programmed cell death [15]. GSH is an important neuronal antioxidant in biological systems that prevents and repairs peroxidative damage to lipids, proteins and nucleic acids. Loss of intracellular GSH is believed to contribute to brain aging and neurodegenerative disorders as reduced neuronal GSH levels are found in AD, PD and other neurodegenerative disorders [16]. Disruption of mitochondrial membrane potential ($\Delta\Psi m$) induced by oxidative stress is regulated by various anti- and pro-apoptotic proteins, such as Bcl-2, Bid, and Bax [17,18]. The pathogenesis of neurodegenerative disorders is induced by multiple factors, such as Ca^{2+} overload, ROS production, lipid peroxidation, mitochondrial dysfunction, and MAPKs activation. Therefore, multi-target strategies are necessary to identify drug leads for neuroprotection or treatment of dementia. BR extract decreased ROS generation and Ca^{2+} influx and increased GSH, GR and GPx activity in HT22 cells. Moreover, BR extract ameliorated mitochondrial dysfunction and showed DPPH radical-scavenging activity. Our findings suggest that BR has a potent neuroprotective effect against glutamate-mediated neuronal cell death, which was associated with an antioxidant effect. BR could be valuable in the multiple-injury neuronal model, and useful for discovery of drugs effective against neurodegenerative disease.

CONCLUSION

The methanolic extract shows significant neuroprotective activity against glutamate induced oxidative cell death in HT22 cells .Based on the results BR extract prevent the oxidative stress by increasing antioxidant levels. So *Biophytum reinwardtii* is a promising therapeutic agent for treatment of neurological diseases like Parkinson's and Alzheimer's. Further studies needed to isolation of bio active compounds and *in vivo* neuroprotective studies.

ACKNOWLEDGMENT

Authors are thankful to management of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur, AP. India and Shri Vishnu College of Pharmacy, Bhimavaram for supporting and providing facilities to conducting this work.

CONFLICT OF INTEREST

No Conflict of interest

REFERENCES

[1]. Albrecht, P., Mechanisms of oxidative glutamate toxicity: the glutamate/cystine antiporter system xc- as a neuroprotective drug target. CNS Neurol Disord Drug Targets, **2010**.9(3):373-382.

[2]. Ankarcrona, M., Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron,**1995**.15(4):961-973.

[3]. Choi, D.W., Glutamate neurotoxicity in cortical cell cultures is calcium dependent. Neurosci Lett, **1985**.58:293-297.

[4]. Duchen, M., Mitochondria and calcium: From cell signaling to cell death. J Physiol, **2000**.529:57-68.

[5]. Hynd, M.R., Scott, H.L., Dodd, P.R., Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. Neurochem. Int, **2004**.45(5):583-595.

[6]. Koga, M., Glutathione is a physiologic reservoir of neuronal glutamate. Biochem Biophys Res Commun, **2011**.409(4):596-602.

[7]. Liu, J., Li, L., Suo, W.Z., HT22 hippocampal neuronal cell line possesses functional cholinergic properties. Life Sci, **2009**.84:267-271.

[8]. Ly, J.D., Grubb, D.R., Lawen, A., Te mitochondrial membrane potential (ψ) in apoptosis; An update. Apoptosis, **2003**.8(2):115-128.

[9]. Markesbery, W.R., Oxidative stress hypothesis in Alzheimer's disease. Free Radic Biol Med, **1997**.23(1):134-147.

[10]. Murphy, T.H., Miyamoto, M., Sastre, A., et al., Glutamate toxicity in neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron,**1989**.2:1547-1558.

[11]. Randall, R.D., Tayer, S.A., Glutamate-induced calcium transient triggers delayed calcium overload and neurotoxicity in rat hippocampal neurons. J Neurosci, **1992**.12:1882-1895.

[12]. Reynolds, I.J., Hastings, T.G., Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. J Neurosci, **1995**.15:3318-3327.

[13]. Sakthivel, K.M., Guruvayoorappan, C., Biophytum sensitivum: Ancient medicine, modern targets. J Adv Pharm Technol Res, **2012**.3:83-91.

[14]. Swerdlow, R.H., Pathogenesis of Alzheimer's Disease. Clin Interv Aging, **2007**.2(3):347-359.

[15]. Tan, S., Wood, M., Maher, P., Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem, **1998**.71:95-105.

[16]. Trease, G. ,Evans, S.M., Pharmacognosy. 15th ed. London: Bailer Tindal,2002, 23-67.

[17]. Weon, J.B., Neuroprotective Effect of Steamed and Fermented Codonopsis lanceolate. Biomol Ter, **2014**.22(3):246-253.

[18]. Yun, B.R., Neuroprotective properties of compounds extracted from Dianthus superbus L. against glutamate-induced cell death in HT22cells. Pharmacogn Mag, **2016.**12(46): 109-112.