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Neuroprotective Effects of Isolated Fraction from *Sapindus laurifolia* Extract in Hippocampal Neuronal HT22 Cells

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Abstract

Glutamate is a major endogenous excitatory neurotransmitter in the brain and contributes to the development of neurodegenerative diseases by excessive activation. The purpose of the present study was to determine the neuroprotective effect of *Sapindus laurifolia* (MESL) Fraction A against glutamate-induced oxidative stress and to assess the underlying mechanism. MESL Fraction A was subjected to a neuroprotective effect assay in HT22 mouse hippocampal cells. The mechanism underlying the neuroprotective effect of MESL Fraction A 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. MESL Fraction A significantly decreased glutamate-induced death of HT22 cells (88.23 ± 1.65% relative neuroprotection). MESL Fraction A reduced the intracellular ROS and Ca^{2+} levels and increased the glutathione level and glutathione reductase and glutathione peroxide activities. Moreover, MESL Fraction A attenuated the mitochondrial membrane potential in HT22 cells. These results suggested that MESL Fraction A exerts a neuroprotective effect against oxidative stress HT22 cells, which was mediated by its antioxidant activity.

Keywords: Antioxidant, Glutamate, Hippocampal Cells, Neuroprotective, Oxidative Stress

1. Introduction

Glutamate is one of the major excitatory neurotransmitter in the brain. It is mainly used for maintenance of memory in synapse, neuronal differentiation and nerve transmission[1]. In case the concentration of glutamate in increased that results in impairment of cognitive functions like loss of learning and memory and is involved in the pathogenesis of neurodegenerative disorders, including Alzheimer's Disease (AD) and Parkinson's Disease (PD)[2]. Two common pathways is involved in glutamate neurotoxicity. In the excitotoxic pathway, which is mediated by ionotropic glutamate receptors? The mechanism of excitotoxicity has been extensively characterized and it is believed that transient Ca^{2+} influxes lead to alterations in calcium homeostasis, increases in the levels of Reactive Oxygen Species (ROS) and ultimately cell deat[3]. The second distinct pathway in glutamate toxicity does not involve glutamate receptors, but rather a glutamate/cysteine antiporter, which is required for the delivery of cysteine into neuronal cells[4]. Inhibition of cysteine uptake by high concentrations of extracellular glutamate leads to an imbalance in cellular cysteine homeostasis, a reduction in cellular glutathione levels and the accumulation of ROS. Glutamate-induced oxidative toxicity has been described in neuronal cell lines, primary neuronal cultures and oligodendrocytes. This oxidative neuronal death pathway is thought to contribute to neuronal injury and degeneration in many MESL in disorders[5]. HT22 cells, an immortalized

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mouse hippocampal cell line, have been widely used as an in vitro model for elucidating the mechanism of oxidative stress-induced neurotoxicity[6]. HT22 cells lack a functional ionotropic glutamate receptor, thus excluding excitotoxicity as a cause of glutamate-triggered cell death. MESL also has anti-inflammatory effects against lipopolysaccharide (LPS)-induced production of NF-κB–dependent inducible Nitric Oxide Synthase (iNOS) and pro-inflammatory cytokines in macrophages. However, MESL has not been reported to be a neuroprotectant that can reduce glutamate-induced apoptosis. Therefore, we examined the protective effect of MESL against glutamate-induced oxidative stress in a mouse hippocampal neuronal cell line.

Sapindus laurifolia also known as little tree plant is a species of plant in the Oxalidaceae family. 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, radio protective, chemo protective, antiangiogenetic, wound-healing, immunomodulatory, anti-diabetic and cardio protective in nature [7].

In present we focused on Neuroprotective effects of *Sapindus laurifolia* Fraction A in mouse hippocampal neuronal cells

2. Materials and Methods

2.1 Plant Material

Sapindus laurifolia fruits was collected from local market of Bhimavaram, West 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, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur.

2.2 Preparation of Plant Extract

Sapindus laurifolia fruits were dried under shade at room temperature the shade dried, coarsely powdered roots (500 g) was successively extracted with petroleum ether (60-80°C) 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 (30°C) to dryness and MESL brownish yellow color extracts of *Sapindus laurifolia* was obtained[8].

2.3 Isolation of Fractions from Extract

2.3.1 Method

Column-chromatography A cylinder shaped glass column containing stationary phase (silica gel) is encountered slowly from the top with a liquid solvent (mobile phase) that flows down the column with the help gravity or external pressure applied. This technique is used for the purification of compounds from a mixture. Once the column is ready, the sample is loaded inside the top of the column. The mobile solvent is then allowed to flow down through the column. The compounds in mixture have different interactions ability with stationary phase (silica gel) and mobile phase, thereby will flow along the mobile phase at different time intervals or degrees. In this way, the separation of compounds from the mixture is achieved. The individual compounds are collected as fractions and analyzed further for structure elucidation.

2.3.2 Protocol

Isolation and purification of bioactive compounds from plant samples.

- A suitable size long cylindrical glass column (based on the amount of the sample) should be stand firm on a column-chromatography stand.
- Completely dried plant extract sample should be mixed with silica gel to make a fine powdered form for easy distribution of sample in already packed silica gel column.
- Sample powdered mass should be placed on the top of the pre-packed silica column and sample should be covered with a layer of cotton.
- Then solvents of different polarities were passed through column at uniform rate under gravity to fractionate the sample extract.
- Each fraction was collected separately in a test tube and numbered consecutively for further analysis on thin layer chromatography.
- Thin Layer Chromatography (TLC) provides partial separation of both organic and inorganic materials

using thin-layered chromatographic plates especially useful for checking the purity of fractions.

- Each fraction is applied on activated TLC plates with the help of capillary tube at a 1/2 inch apart from the lower edge of TLC plate, and plate is kept in a developing chamber containing suitable solvent system for specific time until the developing solvent reaches top of the upper edge of TLC plate.
- Plate is taken out from developing chamber, dried and solvent front is marked by lead pencil. Compound bands/spots visualized on TLC chromatoplate can be detected by visual detection, under UV light (254 nm), in iodine chamber and by using spray reagent (vanillin-sulfuric acid) for the presence of specific compounds.
- The visualized spots of the components in the chromatoplate are marked and the Rf value of each spot is calculated by the formula: Rf = distance travelled by the sample (cm)/distance travelled by the solvent (cm). Table 1.

2.3.3 Preliminary Phytochemical Screening

Different fractions were subjected to preliminary phytochemical for the detection of various constituents.

2.3.4 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 (FMESL), 1% penicillin/streptomycin, $NaHCO_3$ (2 mg/mL), and 15 mM HEPES and were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.3.5 Cell Viability

Cell viability was determined by MTT assay as described previously[9]. 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 MESL Fraction A 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 24 h. After incubation, 1 mg/ml MTT solution was added to each well for 3 h. MTT formazan was Dissolved by Dimethyl Sulfoxide (DMSO) and the optical density at 570 nm was measured using an Enzyme-linked Immunosorbent Assay (ELISA) reader.

2.3.6 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. Tree concentrations of ENS sample were treated with 2 mM glutamate for 8 h in the seeding cells. Simultaneously, 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-buffered saline (PMESL; 0.01 M, pH 7.4) and extracted with 1% Triton X-100 in PMESL (0.01 M, 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.

2.3.7 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 MESL (10 and 100 µg/mL), 2 µM Fura-AM and glutamate. After 20 min, cells were washed with HEPES buffer saline and incubated for 1 h. Fluorescence was monitored at an excitation wavelength of 380 nm with fixed emission at 510 nm.

2.3.8 Measurement of Mitochondrial Membrane Potential (MMP)

MMP was measured using the fluorescent dye Rh123, as reported previously[10]. 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 PMESL. Fluorescence was monitored at an excitation wavelength of 488 nm with fixed emission at 525 nm.

2.3.9 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 MESL. 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.

3. 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.

4. Results

Phytochemical studies of Fraction A (Hexane:Ethyl acetate) revealed the Fraction presence of fattyacids, phenolics and flavonoids. Table No. 1.

Table 1. Gradient solvent system used in column chromatography

Gradient solvent system used in column chromatography for the isolation of fractions in *Sapindus laurifolia* Extract		
Solvent system	**Ratio**	**Fraction**
Hexane	100%	-
Hexane:Ethyl acetate	95:5	A
Hexane:Ethyl acetate	70:30	B
Hexane:Ethyl acetate	50:50	C
Only ethyl acetate	100%	D
Only methanol	100%	E

4.1 Neuroprotective Effect of MESL Fraction A on Glutamate-induced Oxidative Cytotoxicity

MTT assay was performed to investigate the neuroprotective effect of MESL on glutamate-induced death of HT22 cells. We found that 2 mM glutamate treatment reduced cell viability. MESL significantly improved the glutamate-mediated decrease in cell viability in Figure 1. MESL (100 µg/ml) recovered cell viability to 80 ± 0.26% of the control, whereas the viability of the 2 mM glutamate-treated group decreased to 42.17 ± 5.11%. (Figure 2). Trolox, as a positive control, also significantly protected HT22 cells against glutamate-induced cytotoxicity.

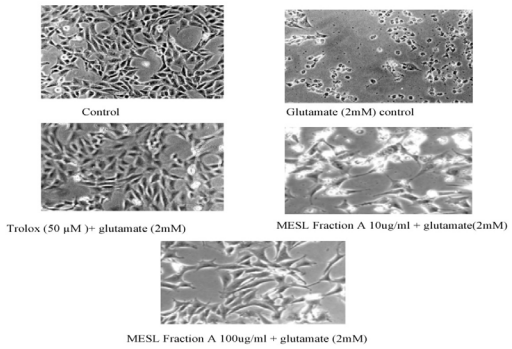


Figure 1. Neuroprotective effect of MESL Fraction A on glutamate-induced oxidative cytotoxicity.

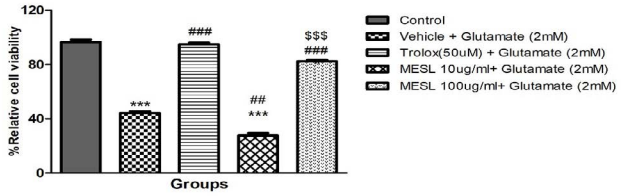


Figure 2. Effect of MESL Fraction A on glutamate induced cell death in hipocampal neuronal HT22 cells. Glutamate is decreased % relative cell viability when compared to normal, where as Trolox and MESL increases % relative cell viability. Values are representing means of triplicate of experiment.***$p<0.01$ significant than normal, ###$p<0.001$ more significant than glutamate treated cells.

4.2 MESL Fraction A Inhibited Glutamate-induced ROS Accumulation

Glutamate-treated cells exhibited increased fluorescence compared to the controls. MESL pretreatment of cells inhibited ROS overproduction in a dose-dependent manner; 100 µg/ml MESL significantly decreased ROS production to 98 ± 0.78% in Figure 3. Therefore, MESL Fraction protected HT22 cells against glutamate-induced death by inhibiting ROS production.

4.3 MESL Fraction A Inhibited Glutamate-induced Ca2+ Influx

We evaluated the effect of MESL on intracellular Ca^{2+} levels in HT22 cells using Fura-AM. As shown in Figure 4, the intracellular Ca^{2+} concentration was increased in

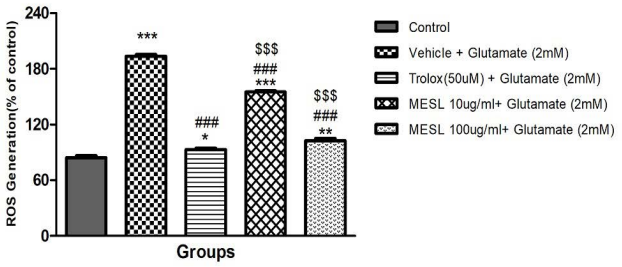


Figure 3. Effect of MESL Fraction A on glutamate induced ROS generation in hippocampal neuronal HT22 cells. Glutamate is increased % ROS generation when compared to normal, where as Trolox and MESL Fraction decreases % ROS generation. Values are representing means of triplicate of experiment. ***p<0.01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

cells treated with glutamate to 170.2 ± 2.56%. However, the Ca^{2+} concentration in cells pretreated with MESL (100 µg/ml) decreased significantly compared with glutamate-treated cells (142 ± 1.85%).

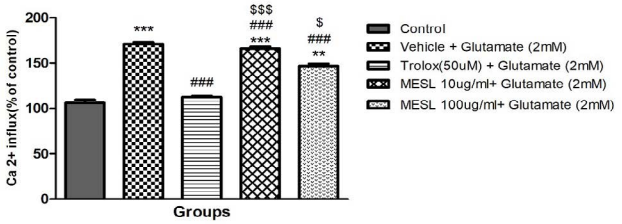


Figure 4. Effect of MESL fraction A on glutamate induced Ca^{2+} influx in hippocampal neuronal HT22 cells. Glutamate is increased % Ca^{2+} influx when compared to normal, where as Trolox and MESL decreases % Ca^{2+} influx .Values are representing means of triplicate of experiment .***p<0.01 significant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

4.4 Inhibition by MESL Fraction A of 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 show that MESL significantly recovered the mitochondrial membrane potential to 112 ± 0.79 % of the control at 100 µg/ml.

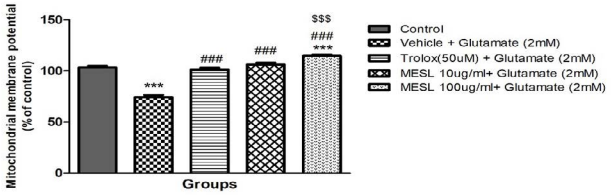


Figure 5. Effect of MESL Fraction A on glutamate induced disruption of mitochondrial membrane potential in hippocampal neuronal HT22 cells. Glutamate is decreased mitochondrial membrane potential when compared to normal, where as Trolox and MESL increases mitochondrial membrane potential. Values are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

4.5 MESL Fraction A Restored GSH, GR and GPx Activities

We confirmed that glutamate-induced death of HT22 cells was related to oxidative stress and the treatment with MESL 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 MESL on GSH, GR and GPx expression levels were similar, as shown in Figure 6. 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, MESL prevented the glutamate-induced depletion of GSH (70 ± 1.5% at 100 µg/ml), MESL (90 ± 0.58% at 100 µg/ml) and GPx (96 ± 0.76%) at 100 µg/ml). These results suggest that MESL exerted a neuroprotective effect, which was mediated by its antioxidant activity.

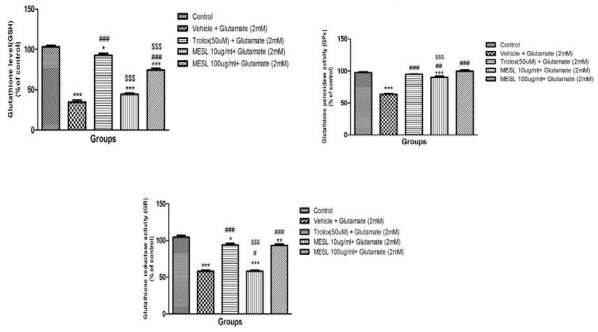


Figure 6. Effect of MESL Fraction A on GSH, GR and GPx activities in hipocampal neuronal HT22 cells. Glutamate is decreased GSH, GR and GPx activity when compared to normal, where as Trolox and MESL increases GSH, GR and GPx activity. Values are representing means of triplicate of experiment. ***p<0.01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

5. Discussion

Oxidative stress elicited by glutamate induces both apoptotic and necrotic death of HT22 cells[11]. 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[12]. Pretreatment with MESL Fraction A 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 per oxidation. Ca^{2+} is an intracellular second messenger in the central nervous system[13]. Increased intracellular ROS levels due to massive Ca^{2+} influx disturb the activity of Lipoxygenases (LOXs) and induce lipid per oxidation and mitochondrial dysfunction, resulting in programmed cell death[14]. GSH is an important neuronal antioxidant in biological systems that prevents and repairs per oxidative 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[15]. 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[16]. 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. MESL Fraction A decreased ROS generation and Ca^{2+} influx and increased GSH, GR and GPx activity in HT22 cells. Moreover, MESL ameliorated mitochondrial dysfunction and showed DPPH radical-scavenging activity. Our findings suggest that MESL has a potent neuroprotective effect against glutamate-mediated neuronal cell death, which was associated with

Table 2. Identification of phytochemical constituents

S.NO	Phytochemical tests	H:EA (95 : 5)	H:EA (70 30)	H:EA (50 : 50)	EA only	H only	M only
1	Alkaloids	-	-	-	-	-	-
2	Glycosides	-	-	-	-	-	-
3	Tannins	-	-	-	-	-	-
4	Phenols	++	-	-	-	-	-
5	Flavonoids	++	++	-	-	-	-
6	Saponins	-	-	-	-	-	-
7	Steroids	++	++				

Where (+) Indicates presence, and (-) Indicates absence
H-Hexane, EA-Ethyl Acetate, M-Methanol.

an antioxidant effect. MESL could be valuable in the multiple-injury neuronal model, and useful for discovery of drugs effective against neurodegenerative disease.

6. Conclusion

The Fraction A from methanolic extract of *Sapindus laurifolia* fruits shows significant neuroprotective activity against glutamate induced oxidative cell death in HT22 cells. Based on the results Fraction A of *Sapindus laurifolia* fruits extract prevent the oxidative stress by increasing antioxidant levels. So *Sapindus laurifolia* 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.

7. Acknowledgment

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8. Conflict of Interest

No conflict of interest.

9. References

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