Neuroprotective evaluation of standardized extract of *Centella asiatica* in monosodium glutamate treated rats

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The effect of chloroform: methanolic (80:20) extract of *C. asiatica* (CA; 100 and 200mg/kg), was evaluated on the course of free radical generation and excitotoxicity in monosodiumglutamate (MSG) treated female Sprague Dawley rats. The extract showed significant improvement in catalase, super oxide desmutase and lipid peroxides levels in hippocampus and striatum regions. Glutathione level was not altered with CA treatment. Similar observation was made with dextromethorphan. The general behavior, locomotor activity and CA1 a region of the hippocampus was significantly protected by CA indicating neuroprotective effect of CA in MSG induced excitotoxic condition. Hence it can be concluded that CA protected MSG induced neurodegeneration attributed to its antioxidant and behavioural properties. This activity of CA can be explored in epilepsy, stroke and other degenerative conditions in which the role of glutamate is known to play vital role in the pathogenesis.

**Keywords:** *Centella asiatica*, Dextromethorphan, Excitotoxicity, Gabapentine, Monosodiumglutamate, Neurodegeneration, Glutamate is the most abundant excitatory neurotransmitter in the brain, released from about 40% of synapses in the central nervous system. Excessive amounts of glutamate may be potent neurotoxin to neurons in addition to its vital role as a neurotransmitter. Although glutamate-induced cell death is associated with both apoptotic and necrotic changes, the mechanism of cell death remains to be established. Two distinct pathways for glutamate-induced cell death have been described: the excitotoxic pathway and the oxidative pathway. The excitotoxic pathway involves the over activation of glutamate receptors that leads to both rapid and slowly triggered cytotoxic events. The rapid effects involve the activation of the N-Methyl-D-Aspartate receptor (NMDAR) that lead to a large Ca\(^{2+}\) influx that may be detrimental to cell viability. The oxidative pathway involves breakdown of the glutamate-cystine antiporter and a drop in glutathione levels which allows for aberrant formation of neurotoxic reactive oxygen species (ROS). NMDAR antagonist and antioxidants were found to inhibit neurodegeneration.

*Centella asiatica* (Umbelliferae, CA) an Indian medicinal plant has been used as analgesics, antiasthmatic, antileprotic, anti-ulcer and anti-inflammatory agents. The herb also exhibited antidepressant activity, improved intelligence, tranquilizing, sedative and anti anxiety effects in experimental animals. CA was effective in preventing the cognitive deficits, as well as the oxidative stress caused by intracerebroventricular administration of streptozotocin, indicating that CA can act as a free radical scavenger. Glutamate play a significant role in neurodegenerative disorders like ischemia, epilepsy and Alzheimer’s disease and one of the mechanisms of glutamate mediated excitotoxicity is through the release of free radicals. Hence controlling the release of glutamate or preventing the glutamate mediated response may be beneficial in above mentioned conditions. CA possesses antioxidant and central nervous system property however the effectiveness of CA in controlling glutamate induced excitotoxicity is not carried out. The present study aims to evaluate the effect of CA on the neurodegeneration induced with monosodium glutamate (MSG) and on oxidative markers in MSG treated rats.

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Materials and Methods

Drugs and chemicals—Gabapentin (Sun Pharmaceutical Industries Ltd., India), dextromethorphan (Dr. Reddy’s Laboratories Ltd., India), bovine serum albumin, NADPH, NADH, tetra ethoxy propane, glutathione reductase were obtained from Sigma Chemicals, USA; DTNB, glutathione, phenazine methosulphate, nitroblue tetrazolinium, thiobarbituric acid, mono sodium glutamate were obtained from Himedia, India

Preparation of the extract—Centella asiatica (CA) was collected from southern part of India and authenticated by Botanical Survey of India, Coimbatore. Herbarium of the plant was prepared and kept in Department of Pharmacognosy, JSS College of Pharmacy, Ooty for future reference. The whole plant was washed, cleaned and shade dried at room temperature. The dried plant was powdered and extracted with 50% ethanol. Enrichment of the 50% ethanol extract was done with chloroform followed by chloroform methanol (20:80) mixture. The solvents were evaporated until a solid residue was formed and stored in desiccator. The yield value of the ethanolic extract of CA was found to be 11%.

Estimation of asiaticoside by HPTLC—Fingerprint of fractionate from CA extract was developed using CAMAG HPTLC system (4.06, Version). Fractionated extract (100mg) was taken and dissolved in 10ml of methanol to get 10mg/ml of sample solution. Solvent system chloroform– glacial acetic acid– methanol– water (60:32:12:8) was used as mobile phase. Ascending chromatogram was developed and scanned at 366nM. The fingerprint of the sample shows 19 peaks, the peak no 9 correspond to the Rf value of asiaticoside (Rf 0.43).

Animals—Adult 4-5 months old female Sprague Dawley rats weighing 200-250 g were used. Animals were procured from the Central Animal House of the Institute and housed in colony cages at an ambient temperature of 25° ± 2° C and 45 – 55 % RH with 12 hr day light / dark cycles. They had free access to pellet chow (Brook Bond, Lipton India) and water ad libitum. Animals were exposed only once to the experiments which were performed between 0900 to 1700 hours. Institutional Animal Ethical Committee under the regulation of Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi approved the project.

Drug treatment—The animals (36) were divided into 6 groups of six animals each. Except vehicle treated group all other groups received mono sodium glutamate (MSG, 2g/kg) intraperitoneally for seven days. The drugs Centella asiatica 100 and 200 mg/kg, gabapentin (20 mg/kg) and dextromethorphan (30 mg/kg) were prepared as fine suspension in 0.3% carboxy methyl cellulose and administered orally for 7 days after one hour of MSG treatment. During the drug treatment the rats were observed for the behavioral changes. On day 8 the rats were evaluated on their ambulatory behaviour. Following the behavioural test on 9th day, the rats were sacrificed and brain was removed for estimation of antioxidant enzymes.

Behavioural test

General behaviour—As an excitatory amino acid glutamate can lead to precipitation of excitation on the animal’s behaviour. To evaluate the excitement and aggressive behaviour the animals were observed for the behavioural changes 45 min after the administration of MSG. Social behavior, hyperactivity, aggression, fighting, sleep, defensive behavior and anticipatory autonomic reactivity were recorded. Rats were scored 5 when they exhibited aggression and fighting behaviour and score 0 for normal social behaviour.

Ambulatory behaviour—The motor activity of the animal was assessed with actophotometer. The animals were kept in the actophotometer and their activity was observed for 10 min.

Biochemical estimations

Tissue preparation—On day nine following the behavioural testing rats were sacrificed by decapitation under ether anesthesia and the brain was quickly removed. Under ice cold condition the hippocampus (HP) and striatum (ST) were dissected, weighed and stored at –70°C. The brain samples were thawed and homogenized with chilled 10% KCl solution (10ml/g tissue) in Elvenjan hand homogenizer. Homogenized samples were centrifuged at 5000 rpm for 10 min. Aliquot was taken for biochemical estimations. Protein concentration was determined following Lowry et al., using bovine serum albumin as standard.

Total glutathione—The following working solutions were made from the stock buffer; (125mM sodium phosphate, 6.3 mM sodium EDTA was adjusted to pH 7.5) 0.3 mM NADPH, 6 mM dinitrothiobisnitroso benzoic acid (DTNB) and approximately 50 units of glutathione reductase per
ml and stored at 4°C. NADPH(700 µl), DTNB(100 µl), glutathione or sample(25 µl), glutathione reductase (10 µl), deionized water (165 µl) were incubated at 30°C and absorbance was read immediately at 420 nm

Catalase (CAT)—Catalase measurement was done based on the ability of CAT to inhibit oxidation of hydrogen peroxide (H₂O₂). Potassium phosphate buffer (65 mM, pH 7.8, 2.25 ml) and 100 µl of the brain homogenate or sucrose (0.32 M) were incubated at 25°C for 30 minutes. H₂O₂ (7.5 mM; 650 µl) was added to initiate the reaction. The change in absorption at 240 nm was measured for 2-3 min. dy/dx for every min for each assay was calculated and the result is expressed at CAT units of protein

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\text{CAT (U) in 100µl of sample} = \frac{dy/dx \times 0.0003}{38.3956 \times 10^{-6}}
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Superoxide dismutase (SOD)—The activity of SOD was estimated following the method of Kakkar et al. One unit of activity of SOD was defined as the amount of enzyme that inhibits the rate reaction by 50% under specified condition. Sodium pyrophosphate buffer (pH 8.3, 0.052M, 1.2 ml), phenazine methosulphate (186 µM), nitroblue tetrazolium (300 µM) and NADH (780 µM, 0.2 ml) were incubated. The reaction was initiated by the addition of NADH; followed by incubation for 90 sec at 37°C. The reaction was terminated by the addition of glacial acetic acid (1 ml), n-butanol (4 ml) was added, shaken vigorously, centrifuged at 4000 rpm for 1 min. upper butanol layer was read at 560 nm against butanol blank.

Thiobarbituric acid reactive substances—Ohkawa et al. method was used to estimate total amount of lipid peroxidation product (thiobarbituric acid reacting substances) in the homogenate. The incubation mixture consists of 0.5 ml of aliquot, 0.2ml of 8% sodium dodecyl sulphate, 1.5ml of 20% acetic acid solution, 1.5 ml of 0.9% aqueous solution of thiobarbituric acid and double distilled water made up to 5.0 ml were heated in boiling water bath for 30 min. After cooling, the red chromogen was extracted into 5 ml of the mixture of n-butanol and pyridine (15.1v/v) centrifuged at 4000 rpm for 10 min. The absorbency of organic layer was taken at 532 nm. 1, 1, 3, 3 tetra ethoxypropane (TEP) was used as an external standard and the level of lipid peroxides was expressed as µmole of MDA/mg protein. The calibration curve of TEP was prepared by the above procedure taking 80-240 nmoles of TEP as standard over which, linearity was obtained.

Histopathological studies
On 9th day, the rats were sacrificed and brain was removed. One half of the brain samples from different groups of MSG treated rats was stored in FAM mixture (40% formaldehyde, acetic acid and methanol in the ratio 1:1:8) for histopathological analysis. Brains were extracted, embedded in paraffin, and sagital sections of 5 µm thickness were used for eosin and hemotoxylin staining to assess the neuroprotective activity of the drugs in CA1 region of the hippocampus.

Statistical analysis—Data are expressed as mean ± S.E. Biochemical data were subjected to one way ANOVA followed post hoc comparison by Newman Keuls test, using GraphPad Prism version 3.00 for windows (GraphPad Software, San Diego, California, USA). A probability levels less than 0.05 has been taken as level of significance.

Results

Behavioural test

General behavior—Treatment of MSG resulted in aggressive behavior and invariably all the animals exhibited hyperactiveness followed by calmness after the administration of MSG. In comparison to MSG (score 3.5±0.75), rats treated with CA (score 2±0.35), gabapentin (score 1.9±0.33) and dextromethorphan (score 1.5±0.53) had significantly decreased hyperactivity indicate the antagonizing effect of these drugs with MSG.

Actophotometer—In comparison to the control rats (75.92 ± 9.2) the ambulatory behavior of the MSG treated rats was significantly decreased (35.46 ± 4.8). Further treatment of CA (55.66 ± 6.8), gabapentin (50.40 ± 7.8) and dextromethorphan (60.70 ± 8.8) significantly increased the ambulatory behavior in rats.

Biochemical estimations

Total glutathione—In comparison to control rats, a significant decrease in the HP and ST glutathione level was observed with MSG treated rats. Treatment of the drugs CA and standard drug dextromethorphan/ gabapentin did not alter the decrease glutathione level in both HP and ST regions (Table 1).

Catalase—Significant decrease in the HP and ST catalase level was found with MSG treatment. Administration of CA (100 and 200 mg/kg) reversed the MSG induced reduction of catalase.
asciatica (200mg/kg) failed to show any effect on HP catalase level. Similarly dextromethorphan significantly attenuated the MSG effect in ST region. GABA agonist gabapentin failed to alter the glutamate effect in both HP and ST regions. (Table 1)

Superoxide dismutase—SOD level was found to be reduced in HP and ST regions of MSG treated rats. Dose dependent increase in SOD level was observed with CA treatment in both HP and ST regions. Dextromethorphan and gabapentin significantly attenuated the MSG effect and increased the SOD level in both HP and ST regions (Table 1).

TBARS—A significant increase in the TBAR level in HP and ST was observed with glutamate treated rats. Treatment of CA, dextromethorphan and gabapentin significantly decreased the TBAR levels. Among the treatments CA (200mg/kg) and dextromethorphan produced better response indicating protective effect of these drugs against MSG induced excitation. (Table 1)

Histopathology studies (Fig 1)

Sagital sections of the brain samples were stained with eosin and haematoxylin to study neurodegeneration of the CA1 region. CA1 region of the control rats was found to be intact and no neuronal loss was observed. CA1 region of the MSG treated rats showed loss of the CA1 structure and demonstrated a corresponding increase in degeneration neurons indicates neurotoxicity of glutamate. Treatment of dextromethorphan and gabapentin significantly protected the neurodegeneration of the CA1 neurons. Similarly administration of Centella asiatica along with glutamate protected the CA1 region however in comparison to standard drugs effect of Centella asiatica was found to be significantly less.

Discussion

One week administration of MSG in rats depleted the glutathione, catalase and superoxide dismutase levels and increased the lipid peroxides in HP and ST regions. The animals also exhibited aggressive behaviour and in some animals fighting behaviour was also observed. Further loss of hippocampal structure was noted in MSG treated rats. These results clearly show that glutamate in rats can lead to excitation and oxidative stress resulting in neurodegeneration. Treatment of standardized CA, herb containing asiaticosides significantly attenuated the glutamate induced excitation and oxidative stress. The present findings are in accord to the earlier report demonstrating antioxidant property of CA in the conditions like intracerebroventricular streptozotocin model of Alzheimer's disease in rats, lymphoma-bearing mice and adriamycin induced cardiomyopathy in rats.

In glutamate toxicity the neuronal death is linked closely to glutamate-evoked excitotoxicity. Glutamate plays a central role in neurodegeneration, and increases extracellular glutamate concentrations from 30 to 200 μM in ischemic brain. A characteristic response to glutamate challenge is the increase in the cytosolic Ca2+ level, which is due to either influx from the extracellular space or release from the intracellular stores. In this excitotoxic condition the survival of a cell depends largely on functioning of the mitochondria. The mitochondrial potential, the driving force necessary to satisfy the cellular energy demands, is also involved in the reactive oxygen species (ROS)
generation, which in turn are suspected to cause cell death if they get out of control. The interplay between mitochondrial potential and ROS generation is not yet fully understood\(^2\). Since CA could control the neurodegeneration and elevated the antioxidant enzymes it can be stated that CA produced antioxidant properties in this model of excitatory response through controlling the glutamate induced radical generation.

Further, the reversal of MSG induced behavioural alteration with CA may be attributed to its central GABA activity\(^3\). Mohandas Rao et al\(^{25}\), have demonstrated that administration of fresh leaf extracts of CA during the growth spurt (neonatal) period facilitated the dendritic growth in the hippocampal CA3 neurons. In addition to its effect on behavior, the active components of the CA, asiatic acid, asiaticoside
and SM2, showed neuroprotective property and inhibited beta amyloid and free radical induced cell death in B103 cell cultures and hippocampal slices. All these reports clearly show that CA can act as a neuroprotective agent and supports our present study. These effects of CA were comparable with dextromethorphan.

Dextromethorphan increased the superoxide dismutase, catalase and decreased TBAR levels in hippocampus and striatal regions. This effect of dextromethorphan may be attributed to their antagonizing activity at NMDAR, leading to controlling the glutamate excitotoxicity and preventing the free radical generations resulting in the preservation of brain antioxidant enzymes. In comparison to dextromethorphan, gabapentin failed to show much antioxidant properties which indicates that activation of NMDAR would have played role in glutamate mediated effect. Though GABA has shown better behavioural response through their inhibitory mechanism it failed to control the degeneration induced with MSG.

Another interesting observation made in the present study was depletion of glutathione levels with glutamate treatment. Though the treatment of CA, dextromethorphan, gabapentin increased few or all antioxidant enzymes in brain, they all failed to show any effect on glutathione levels. It has been stated that synthesis of glutathione in astrocytes involves glutamate, cysteine and glycine. Excess of glutamate will inhibit the transport of cysteine there by blocking the glutathione formation\textsuperscript{26}. Further it is also reported that the oxidative pathway involves the breakdown of the glutamate-cystine antiporter leading to decrease in glutathione levels that allows for aberrant formation of free radicals that are neurotoxic\textsuperscript{4,5}. In the present work administration of MSG (2g/kg) would have blocked the synthesis of glutathione and resulted in the depletion of glutathione invariably in all the groups.

Hence it can be concluded that CA protected MSG induced neurodegeneration attributed to its antioxidant and behavioral properties. The study also suggests that the protection of antioxidant enzymes activity along with the direct antagonism of glutamate receptor may be beneficial. This activity of CA can be further explored in stroke, epilepsy and other degenerative conditions in which the role of glutamate was known to play vital role in the pathogenesis.

References


